Synthetic Gene Circuits

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Keywords

Analog circuit:
a circuit that produces a graded response based on the levels of inputs it receives. Analog circuits can have a graded transfer function over a wide range of input concentrations.

Digital circuit:
A circuit that produces an all-or-none response, depending on whether its inputs are above or below a defined threshold level. Digital circuits include digital logic gates. Complex circuits may include layered logic gates, so that the output of one gate provides the input for another.

Logic gate:
A device that accepts inputs in the form of TRUE and FALSE (also represented as 1 and 0, respectively) and returns a single TRUE or FALSE output based on Boolean logic

operations such as AND NOT OR, and others. For example, an AND gate returns the TRUE output if, and only if, all inputs are TRUE.

Orthogonality:
The ability of circuit components to function in the same cell without crosstalk; for example, two transcription factors that bind distinct DNA motifs, or RNA molecules that regulate distinct transcripts. Orthogonal components are vital for building complex synthetic circuits.

Oscillator:
A circuit that cycles repeatedly between states, such as high and low levels of expression of a particular protein; one of the prototypical examples of a synthetic gene circuit.

Synthetic transcription factor (sTF):
A human-made transcription factor designed to regulate transcription from a specific promoter; often designed to be responsive to an input, such as a small molecule. May include domains from naturally occurring transcription factors, such as a transactivation or ligand-binding domain, and rationally designed motifs, such as sequence-specific DNA-binding zinc fingers.

Toggle switch:
A circuit that can exist in one of two stable states and may be switched (toggled) between the two states by a defined input; along with the oscillator, the toggle switch is a classic example of a proof-of-principle synthetic gene circuit.

Transfer function:
The output level of a gene circuit as a function of the input level(s) (e.g., the activity of the fluorescent reporter output as a function of the concentration of a small molecule input).

Tunability:
The ability to adjust the activity level of a synthetic circuit component, such as the strength of gene expression from a synthetic promoter.

The past decade has witnessed tremendous advances in the design and implementation of synthetic gene circuits that program living cells to perform specific user-defined tasks. Synthetic circuits have been implemented in bacteria, yeast, and mammalian cells, using a variety of transcriptional and post-transcriptional regulatory mechanisms. These devices, which lie at the intersection of biology and engineering, have provided insights into the function of naturally occurring

gene regulatory networks. Furthermore, they hold the potential for transformative future applications in medicine, bioremediation, manufacturing, and more. In this review, some examples are presented of commonly used synthetic circuits, including oscillators, switches, memory devices, and circuits that perform digital and analog computation. The building blocks of synthetic gene circuits, as well as the challenges and considerations of circuit design, are also discussed. Finally, an overview is provided of the potential practical applications of this dynamic field of research.

1 Introduction to Synthetic Gene Circuits

Living cells monitor their environment and respond to a variety of inputs with sophisticated behaviors, including changes in gene expression, cell morphology and motility, regulation of the cell cycle and growth, and protein secretion. The genetic circuits that control these behaviors have been selected over evolutionary time to enhance the fitness of the cell (or multicellular organism). The emerging and rapidly expanding discipline of synthetic biology aims to engineer synthetic genetic circuits that perform user-defined functions in a predictable and reliable manner.

The synthetic biology approach is modeled explicitly after other engineering disciplines, such as electrical and mechanical engineering. Like other forms of engineering, synthetic biology aims to adopt different levels of abstraction for design, from a very general, high-level description of desired circuit behavior (e.g., ''design a circuit that recognizes and kills cancer cells''), to a detailed description of the molecular mechanisms used to implement the desired behavior, including the sequences of all the DNA-based components.

The motivations for building synthetic gene circuits include both discovery and applications. Early examples of synthetic circuit design include relatively small and simple genetic devices that were used to study the principles underlying the behavior of gene regulatory networks (for reviews, see Refs [1–4]). More recent studies have rewired the native regulatory pathways of living cells in order to study the design principles of gene networks in a ''learn by design'' approach [5], as well as to gain insights into complicated processes such as malignant transformation [6]. Meanwhile, many synthetic biologists are aiming to build circuits with possible practical applications in areas such as medicine, environmental bioremediation, the manufacture of biofuels and valuable chemicals, and biological computation [1, 7].

The review begins with a summary of the building blocks of synthetic gene circuits (Sect. 2). In Sections 1–7 are presented a number of commonly used and studied types of synthetic gene circuit, including oscillators, toggles and cascades (Sect. 3); memory devices (Sect. 4); digital and analog circuits (Sects. 5 and 6); and multicellular systems (Sect. 7). While synthetic biology has progressed greatly during the past few years, significant challenges remain, and these are discussed briefly in Sect. 8. The review concludes with a survey of applications of synthetic circuits (Sect. 9) that provide an overview of the potential for the tremendous advances in technology and medicine offered by this burgeoning field of research.

2
Building Blocks of Synthetic Gene Circuits

A genetic circuit often consists of three parts: (i) a sensor, which accepts an input or inputs; (ii) a processor, which computes the desired response to the input(s); and (iii) an actuator, which produces the corresponding output. The function of a synthetic gene circuit depends on its building blocks (synthetic DNA, RNA, and proteins), as well as the way these components are wired together into sensor, processor, and actuator modules. The building blocks of synthetic gene circuits may be rationally designed or harvested from Nature, sometimes accompanied by directed evolution to alter their performance in a desired way.

In this section, the choice of host cells for implementing synthetic circuits (the chassis), circuit inputs and outputs is discussed, as well as the molecular implementation of the circuits themselves. Attention is focused mainly on transcriptional control, which has been used extensively in synthetic gene circuits; RNA- and protein-based approaches are also briefly discussed. Considerations of circuit topology are provided in Sect. 8.

2.1
The Chassis: Choice of the Host Cell

Almost all synthetic gene circuits developed to date have been implemented in the bacterium *Escherichia coli*, the budding yeast *Saccharomyces cerevisiae*, or mammalian cells. Each of these hosts presents a unique set of advantages and challenges, as well as distinct host–circuit interactions that must be considered when designing circuits for operation in living cells.

Many of the earliest synthetic circuits were implemented in the model bacterium *E. coli* [8, 9] (see Sect. 3). The advantages of *E. coli* include its relatively small genome, extensive toolbox for genetic manipulation, rapid and easy growth characteristics, and simple and well-understood manner of transcriptional regulation. In addition to providing a fertile testing ground for proof-of-principle genetic circuits, *E. coli* is also an interesting organism for practical applications in bioremediation; for the manufacture of biofuels, pharmaceuticals, and other valuable chemicals; and for human health (e.g., identifying mechanisms to combat antibiotic-resistant pathogenic bacteria, or engineering bacteria to find and destroy cancer cells).

The budding yeast *S. cerevisiae* presents an excellent model system for designing synthetic circuits in eukaryotes (for a review, see Ref. [10]). Like *E. coli*, *S. cerevisiae* is quick and easy to grow in the laboratory, and it offers a well-developed suite of genetic tools, including the ability to maintain foreign genetic elements stably on plasmids and to achieve an efficient homologous recombination of synthetic constructs into the genome. At the same time, *S. cerevisiae* offers a variety of useful characteristics for designing logic circuits that bacteria lack, including intracellular compartmentalization and a rich regulatory repertoire with complex transcriptional regulation and protein signaling cascades. Therefore, yeast can be thought of as a "testing platform" for synthetic biology approaches that can then be adapted to more complicated mammalian cells [11]. *S. cerevisiae* is also a workhorse of industrial synthetic biology, and there is great interest in programming yeast strains for improved production of biofuels and commodity chemicals [12].

Mammalian cells are highly desirable targets for synthetic circuit engineering, due to a myriad possible applications such as therapeutics and tissue engineering

[13]. However, these cells have highly complex functions, gene regulation, and intercellular interactions. Furthermore, it is more difficult to work with mammalian cells compared to microorganisms in many technical aspects, such as culture maintenance, DNA delivery, and experimental turnover time. Thus, compared to yeast and bacteria, mammalian cells pose a significantly greater challenge for synthetic biologists. Nonetheless, the past few years have seen many advances in programming circuits in mammalian cells, as described below. Popular mammalian cell types that are well adapted to laboratory conditions include HeLa (human cancer), HEK293 (human embryonic kidney), and CHO (Chinese hamster ovary) cells.

2.2 Inputs and Outputs of Synthetic Circuits

Many synthetic gene circuits are designed to perform a specific action in response to a defined input. Commonly used inputs during circuit design are convenient small molecules that cells can be engineered to respond to, including antibiotics such as anhydrotetracycline (aTc); metabolites or their analogs, such as arabinose or isopropyl β-D-thiogalactopyranoside (IPTG), which mimics a lactose metabolism intermediate; and acyl homoserine lactones (AHLs), diffusible molecules that bacteria use to communicate with each other (see Sect. 7.1) [14–16]. In Nature, bacteria respond to such inputs by activating or repressing target gene expression; for example, arabinose triggers the transcription of genes that encode enzymes and transporters needed to utilize this sugar [17]. Synthetic biology rewires the cell's response to these small-molecule inputs. Cells can also trigger gene expression in response to an external stressor, such as a pulse of heat or DNA-damaging ultraviolet radiation, and these stimuli have been used as inputs to synthetic gene circuits [9, 18]. Their disadvantage, however, is that they can stimulate wide-ranging responses in the host cell that may interfere with desired function of the circuit, and prolonged or repeated exposure may kill the host cell.

More recently, various research groups have harnessed specific wavelengths of light as inputs into synthetic gene circuits, for example, by using light-sensitive proteins from photosynthetic or light-sensitive organisms, including cyanobacteria and plants [19, 20]. One of the advantages of light over a diffusible small molecule is the exquisite level of spatiotemporal control that it offers; it is possible to shine a light specifically onto one part of a plate covered with engineered cells. Moreover, light can be switched on and off instantly, unlike a small molecule which, once applied, can only be removed through washing or gradual dilution. The field of controlling cell behavior with light, named optogenetics, has undergone explosive growth during the past few years (for a review, see Ref. [21]).

Lights: used to control cell behavior.

While many proof-of-principle synthetic circuits use exogenous small molecules or other inputs that are easy to measure and apply, synthetic biology also aims to create circuits that respond to endogenous and functionally relevant inputs, such as disease markers [22–24]. Designing a circuit to respond to such input is challenging, particularly because of the challenge in finding or designing relevant sensors [13, 22–24]. However, a number of circuits responsive to endogenous inputs have been constructed (see Sect. 9).

The outputs of synthetic gene circuits are frequently fluorescent reporter proteins, because such outputs are easy to detect and quantify, enabling

characterization of circuit performance and dynamics. Flow cytometry allows several different fluorescent species to be quantified per cell in a high-throughput manner, and time-lapse microscopy allows the observation of changes in output levels in response to defined inputs in single cells over time. Hence, fluorescent proteins are frequently used in proof-of-principle demonstrations as well as for troubleshooting circuits; subsequently, once the circuit displays a desired performance based on fluorescence assays, the reporter genes may be replaced by output genes that perform a desired function.

Future circuits will increasingly aim to produce outputs relevant for industrial or therapeutic applications, such as control over the cell's proliferation, survival, differentiation, morphogenesis, migration, or synthesis and secretion of a therapeutic protein or commodity chemical. Examples of circuits with functionally relevant outputs are described in Sect. 9.

2.3 Properties of Synthetic Building Blocks

The function of a synthetic circuit depends upon the function of its components (for reviews, see Refs [1] and [2]). Synthetic building blocks, such as transcription factors (TFs) or RNA regulatory elements, should ideally be:

- *Modular:* The component should have a defined function that persists regardless of context; for example, a promoter drives the expression of a downstream gene at the same level, regardless of the identity of the downstream gene. Modularity is the property that allows basic building blocks to be assembled into complex devices in a predictable manner.
- *Composable:* The components should be arranged together into a functional circuit. The output of one part of the circuit can serve as the input for a downstream part.
- *Orthogonal:* The components should avoid unwanted crosstalk with each other, or with the host cell's molecular machinery. Unlike an electronic device, in which components are connected by physical wires, a gene circuit consists largely of building blocks that freely diffuse and mix inside the cell, creating the potential for unwanted interactions. For example, if a circuit includes the TFs A and B that regulate promoters P_A and P_B, respectively, then the unwanted regulation of P_A by B may lead to circuit failure. Likewise, unplanned interactions between a synthetic factor and the cell's native genes may disrupt circuit function or harm the host cell.
- *Tunable:* Different circuit functions require components with different levels of activity, such as promoters that drive expression of downstream genes at different levels. Moreover, successful circuit design requires level matching between upstream and downstream parts of the circuit (see Sect. 8.1). Hence, synthetic biology requires components that display tunability – the ability to achieve different output levels through small changes, such as point mutagenesis of a promoter or a RNA regulatory device.

Orthogonality and tunability require the construction of large libraries of each type of circuit component, such as promoters, TFs, ribosome-binding sites (RBSs), regulatory RNA molecules, and others. Efforts have been made to construct libraries of orthogonal parts with reduced unwanted

crosstalk [25–27]. Libraries of building blocks may be obtained through a combination of three approaches [28]: (i) parts mining, which involves searching the annotated genomes of different species for components that are predicted to have the desired function while avoiding crosstalk with the host cell; (ii) directed evolution, whereby a library of mutagenized components is constructed and subjected to selection *in vitro* or *in vivo* in order to identify components with desired activity; and (iii) rational design, which is applicable to the types of device that operate according to well-understood rules. For example, synthetic transcription factors (sTFs) based on transcription activator-like effectors (TALEs) offer a way to obtain orthogonal TFs by rational design [29] (see below).

It is possible to use a combination of rational design and directed evolution to achieve tunability and to produce libraries of components with the same function, but with different activity levels. For example, the expression level of a protein depends partly on the sequence of the RBS on its corresponding mRNA; hence, protein expression levels can be adjusted through point mutations in the RBS. The RBS calculator [30] is a program for rationally designing an RBS sequence that results in a user-defined protein expression level. Similarly, promoters can be tuned via point mutations [31]. In addition, some circuits are designed to be tunable *in vivo* through the addition of small-molecule inputs. For example, a bandpass filter has been designed that allows its host cells to survive only at a specific concentration of two antibiotics, ampicillin and tetracycline [32]. Varying the levels of the inducer IPTG changes the target concentration of ampicillin and tetracycline at which cells can survive by adjusting the expression of the bandpass filter's components [32] (see Sect. 3.4).

Below are described several of the most commonly used families of synthetic circuit components (also see Table 1 in Ref. [1]). Some examples of commonly used synthetic circuit building blocks are shown in Fig. 1.

2.4 Building Blocks of Synthetic Transcriptional Regulation

Transcription is a key mechanism for gene regulation in living cells. In its most basic form, a transcriptional unit consists of a TF and its cognate promoter/regulator. The TF binds its regulator region in a sequence-specific manner and activates or represses its transcription, in some cases in response to an input such as a small molecule. The modular nature of eukaryotic TFs enables the construction of sTFs by combining different DNA-binding, ligand-binding, and regulatory domains [26, 31, 33–36]. Some transcriptional regulatory domains, such as the commonly used viral VP16 activation domain or its derivative, the VP64 domain [37], activate gene expression from their target promoter. In this case, the cognate synthetic promoter is either a very weak or a minimal promoter located downstream of the TF recognition site, and transcription is initiated from this promoter in the presence of the TF. Other transcriptional regulatory domains inhibit gene expression, such as the Krüppel-associated box (KRAB) domain found in vertebrates [38], which inhibits transcription by recruiting chromatin-modifying proteins that cause formation of repressive heterochromatin. In this case, the cognate synthetic promoter is usually a strong constitutive promoter with associated TF recognition

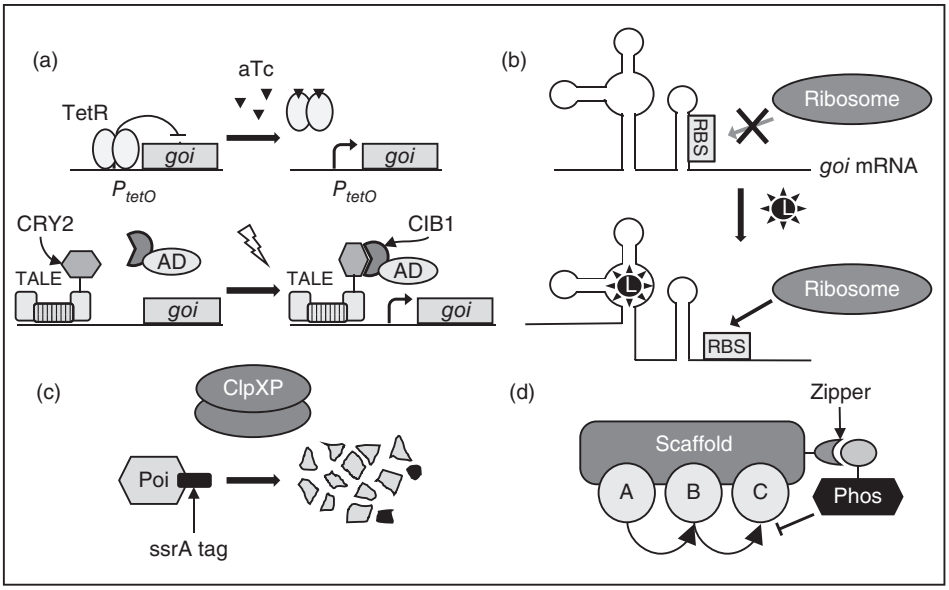

Fig. 1 Examples of commonly used synthetic building blocks. (a) Transcriptional regulation is the backbone of many synthetic circuits (see Sect. 2.4). Top: The bacterial TetR transcriptional repressor forms homodimers that bind tightly to their DNA target motifs, sterically blocking RNA polymerase recruitment to the PtetO promoter. Binding of the small molecule anhydrotetracycline (aTc) causes TetR to dissociate from DNA, allowing expression of the downstream gene of interest (goi). TetR-based transgene regulation is commonly used in *E. coli* and has also been adapted for use in eukaryotes. Bottom: Transcription Activator-Like Effectors (TALEs) bind to user-defined DNA sequences. Here, a synthetic TALE-TF is used to activate a goi in mammalian cells in response to blue light [39]. Upon illumination, the light-sensitive CRY2 domain fused to the TALE-TF undergoes a conformational change, resulting in recruitment of its partner CIB1 domain along with a transcriptional activator domain (AD) that stimulates target gene expression. Lower panel adapted from Ref. [39]; (b) RNA-based devices can regulate target gene expression at different levels, including transcriptional elongation, translation initiation, and mRNA stability (see Sect. 2.5). In this example, an aptamer (ligand-binding RNA molecule) is fused to an mRNA encoding a transgene of interest. In the absence of ligand (top), the aptamer folds into a stem-loop structure that blocks the ribosome binding site (RBS), preventing mRNA translation. The ligand (L) causes a conformational change in the aptamer, exposing the RBS and allowing translation (bottom). Adapted from Ref. [40]; (c) Regulated protein degradation can be used to control synthetic circuit activity at a post-translational level (see Sect. 2.7). Here, an ssrA tag fused to the protein of interest (Poi) targets the protein for degradation by the ClpXP protease. Adapted from Ref. [41]; (d) Synthetic fusion proteins can ''rewire'' intracellular signaling cascades. Here, a scaffold protein brings together three protein kinases (A, B, and C), resulting in efficient signal propagation in an *S. cerevisiae* Mitogen Activated Protein Kinase (MAPK) pathway. This naturally occurring system is ''rewired'' by fusing the scaffold to a leucine zipper domain, which recruits a protein phosphatase (Phos) to the signaling complex [42]. The phosphatase attenuates MAPK signaling by dephosphorylating and hence inactivating the MAP kinase. In contrast, the recruitment of an activating protein to the scaffold potentiates MAPK signaling (not shown) [42]. Adapted from Ref. [42].

sites that is active by default until silenced by the TF. Depending on its mechanism of action, the same sTF may activate or repress transcription of a target gene, depending on where in the gene it binds [43].

In bacteria, transcriptional repressors downregulate gene expression at close range, via directly interfering with RNA polymerase (RNAP) or activator binding [44]. Transcriptional repression in *S. cerevisiae* also tends to occur over short distances due to the compact nature of the genome, and may involve interference with the recruitment of basal transcriptional machinery as well as chromatin-mediated silencing [45, 46]. In contrast, mammalian repressors often involve chromatin remodeling and can have long-range effects of up to tens of kilobases both upstream and downstream of their binding sites [47]. Thus, transgenes inserted into the mammalian genome may become silenced by repressive chromatin spreading from adjacent loci, a phenomenon that may be countered by flanking the transgene with insulator sequences [48]. Furthermore, chromatin remodeling is time-consuming, resulting in slow kinetics of transcriptional regulation in mammalian cells [49].

As mentioned in Sect. 2.3, one of the key requirements of good synthetic building blocks is orthogonality: the ability of multiple elements to exist in a single cell without crosstalk. The regulatory domain can be the same among multiple sTFs operating in parallel without impairing orthogonality. However, off-target binding of the sTF to DNA might result in an unwanted deregulation of native genes and shunting of the sTF away from its target promoter. Thus, the DNA-binding domain of an sTF must be highly specific and target DNA sequences that are very rare or absent in the host genome. This is a greater challenge for mammalian cells than for bacteria and yeast, due to the difference in genome sizes (3×10^9 bp in humans [50] compared to $\sim 5 \times 10^6$ in *E. coli* [51] and $\sim 12.5 \times 10^6$ in *S. cerevisiae* [52]).

The development of sTFs has occurred in stages, with each new generation of sTFs providing new ways to improve orthogonality and tunability. The ''first-generation'' synthetic transcriptional circuits repurposed TFs that occur naturally in bacteria or in phages (viruses that infect bacteria). Examples include the LacI, TetR, and λ CI TF-promoter pairs [8, 9], which were initially used to build synthetic circuits in bacteria and later optimized for use in eukaryotic cells (Fig. 1a). Transcriptional repression by LacI and TetR is relieved by binding to the small molecules IPTG and aTc, respectively; hence, these TFs can be used to build synthetic gene circuits that respond to small-molecule inputs [8, 9]. Early experiments in mammalian cells utilized the DNA-binding domain of the *S. cerevisiae* GAL4 TF, which recognizes an upstream activating sequence (UAS) [53]. The GAL4 DNA-binding domain fused to the VP16 transcription activation domain (VP16AD) can activate gene expression from a UAS-containing cognate target promoter [37]. This useful system exhibits tight regulation and a wide dynamic range.

In order to control the level of transcription output in response to a small-molecule input, ligand-inducible systems were further developed in mammalian cells. The most-characterized system is based on the bacterial tetracycline-dependent transactivator (tTA) that, in its native form, binds its target DNA motif in the absence of tetracycline. Thus, a tTA–VP16AD fusion was utilized for the construction of a tetracycline-off

system, which activates its target gene in absence of tetracycline [38]. Later, tTA was mutated to generate a reverse tetracycline-dependent transactivator version (rtTA), which binds its target site only in the presence of the tetracycline analog doxycycline (Dox), constituting a tetracycline-on system when fused to VP16AD [54].

An alternative ligand-responsive transcriptional regulatory system was based on mammalian nuclear hormone receptors, TFs that regulate target genes in response to steroid hormones such as estrogen and progesterone, which diffuse inside the target cell and bind to the hormone receptor's ligand-binding domain (LBD), enabling regulation of the target gene [55]. The simplicity of such systems, which do not require an elaborate signal-transduction pathway inside the host cell, make LBDs attractive for engineering ligand-responsive sTFs by fusing LBDs to DNA-binding and regulatory domains of choice for use in mammalian cells [56–58] and in *S. cerevisiae* [59]. To avoid crosstalk with the mammalian host cell's native hormone signaling, LBDs have been engineered to respond specifically to synthetic compounds, such as the progesterone antagonist RU486 [57]. Alternatively, LBDs responsive to insect hormones such as ecdysone may be used [56]. Additional ligand-dependent mammalian sTFs have been developed to respond to a variety of ligands such as macrolides [60] and streptogramins [61], L-arginine [62], biotin [63], urate [24], and others [64].

Although well-characterized TFs based on naturally occurring proteins such as bacterial TetR or yeast GAL4 have many advantages, generating a library of sTFs sufficient for building complex gene networks requires another approach. The "second-generation" sTFs have custom-designed DNA-binding domains that can bind any user-defined DNA sequence with high specificity. The first attempt to develop such sTFs focused on engineering the DNA-binding domain of zinc finger transcription factors (ZF-TFs) [33, 65, 66]. A zinc finger is a small protein domain of about 30 amino acids that recognizes a specific 3 bp DNA sequence [67]. A synthetic protein that includes a tandem array of zinc fingers will specifically bind to a user-defined sequence in the genome [68], circumventing the need to rely on the small number of DNA-binding domains found in naturally occurring TFs. Pioneering studies demonstrated that synthetic ZF-TFs can regulate endogenous human genes in a sequence-specific manner [69]. More recently, suites of orthogonal ZF-TFs have been constructed for use in synthetic gene circuits in mammalian cells [70] and in *S. cerevisiae* [26]. The design of custom ZF-TFs was facilitated by publicly available online tools such as the Zinc Finger Targeter (ZiFiT) that identifies potential ZF-binding sites in user-supplied DNA sequences [71], as well as the publicly available Zinc Finger Database (ZiFDB) that provides information on functional zinc fingers [72]. Fusing ZF DNA-binding arrays to the LBD of a nuclear hormone receptor produced ZF-TFs that regulate their targets in response to hormone inputs [73, 74].

Although they represented a great technical advance, ZF-TFs are not trivial to design, and in some cases can lack specificity. For example, adjacent zinc fingers can influence each other's binding specificity, thus complicating the design of tandem arrays of these domains [75]. An alternative approach utilizes the TALE proteins from *Xanthomonas* spp. bacteria, which are plant pathogens that use TALEs to modulate their host cell's gene expression [29, 76].

TALEs feature arrays of short amino acid repeats, with each repeat specifically binding a single DNA base pair. Hence, similar to ZF-TFs, synthetic TALEs can be programmed to bind a specific DNA sequence by combining specific base-pair-binding amino acid sequences in the correct order [29, 77]. Furthermore, the individual TALE domains are relatively independent of one another, enabling modular assembly into higher-order arrays to target longer DNA sequences. Custom-designed TALE-TFs have been used to activate reporter genes in mammalian cells [34, 76]. Although TALEs naturally act as transcriptional activators, targeting TALE-TFs to the core promoter region of target genes in order to block RNAP recruitment can turn the TALE-TFs into transcriptional repressors, as shown in *S. cerevisiae* [31]. Like ZF-TFs, TALE-TFs can also respond to hormone-mediated induction when fused to LBDs of nuclear hormone receptors [78]. Another recent study presented TALE-TFs that regulate genes of interest in response to blue light (Fig. 1a) [39]. The system consisted of two parts: a TALE DNA-binding domain fused to the *Arabidopsis thaliana* light-sensitive cryptochrome 2 (CRY2) protein, and the CRY2 binding partner CIB1 fused to a transcriptional effector domain, such as VP64. When illuminated by blue light, CRY2 recruits CIB1, leading to the formation of a functional TF that can regulate the gene of interest. The system displays high specificity and signal-to-noise ratio, as well as faster response time compared with small-molecule-inducible TALE-TFs [39].

In addition to using TALE-TFs to regulate target genes via regulatory domains such as VP16, recent studies have harnessed TALEs for targeting chromatin modifications to endogenous loci of interest. For example, TALEs fused to the TET1 DNA demethylase or the LSD1 histone demethylase allow site-specific DNA or histone demethylation in mammalian cell culture [79, 80]. Zhang and colleagues used the light-sensitive TALE system described above [39] to target chromatin modifications to specific genes in response to light by fusing the CIB1 protein to various histone-modifying enzymes. This ability to modify the host cell's chromatin at selected loci has exciting implications for future research in areas such as cell fate specification and maintenance, as well as cancer.

A recent breakthrough in TF engineering came from the bacterial CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system, in which sequence specificity can be easily determined by the guide RNA (gRNA) sequence rather than protein engineering [81]. The CRISPR system serves as a form of immune memory in many Bacteria and Archaea, as it causes the cell to cleave the DNA of viruses it has previously encountered and ''remembered'' by incorporating viral sequences into its CRISPR locus [82]. Short RNAs guide a Cas (CRISPR-associated) endonuclease to cleave DNA that complements the RNA sequence. The results of recent studies have shown that the Cas9 protein from *Streptococcus pyogenes* and synthetic gRNAs can cleave specific DNA sequences when expressed in heterologous hosts such as *E. coli* [83] and mammalian cells [84, 85]. The CRISPR system presents an attractive, efficient method for reprogramming cells by genome engineering (e.g., knocking out a specific gene of interest) [84, 85]. In addition, it was shown that a deactivated Cas9 protein that lacks endonuclease activity (abbreviated dCas9) can be recruited by its associated gRNAs to specific DNA loci,

where it can act as a transcriptional regulator without cleaving the target DNA [43, 81]. Once recruited to the locus of interest, dCas9 can repress gene expression by sterically inhibiting RNAP binding to the promoter; alternatively, dCas9 fused to a transcriptional coactivator domain such as VP16 can activate target gene transcription [43, 86]. The potential advantages of the CRISPR system over ZF-TFs and TALEs include a greater ease of design, as sequence-specific gRNAs are easier to design than are zinc fingers or TALE motifs, as well as the possibility of building a circuit with many orthogonal parts. In the ZF-TF and TALE systems, each target promoter requires a separate regulatory protein, while in the CRISPR system, a single dCas9 protein may regulate multiple genes by associating with distinct gRNAs, although this configuration may present problems concerning the allocation of a fixed resource (the dCas9 protein) among multiple gRNAs targeting different promoters.

In order to facilitate the design of sTFs based on zinc fingers, TALEs, and CRISPR, a recent study presented a set of formal rules for building functional sTFs based on 11 possible architectures [35]. Besides considering the sTF architecture, the sTF must also be designed to target a DNA motif that is not expected to cause unwanted crosstalk with the host cell's native genes. To this end, Lu and colleagues [36] identified 9-, 12-, and 15-bp DNA motifs that are underrepresented in or absent from the genomes of six model microorganisms, including *E. coli* and *S. cerevisiae*. This data set can aid in the design of orthogonal promoters for synthetic gene circuits. Another study identified over 180 DNA sequences, each of 20 bp, that differed by at least 3 bp from all possible 20-mers in annotated human promoter regions [34]. Altogether, recent advances in orthogonal sTFs present a resource of great value for building synthetic regulatory circuits.

2.5 Post-Transcriptional Regulation: RNA-Based Circuit Engineering

While most synthetic circuits rely on transcriptional regulation, RNA-based regulatory devices are also common, due to the many attractive features of RNA regulatory molecules (for reviews, see Refs [87, 88]). First, the rules for rational construction of RNA devices with desired function are relatively well understood, enabling the construction of libraries of orthogonal RNA components. Rules for the rational development of synthetic RNA devices with desired properties have been described, such as the theoretical framework for the development of aptazymes [89] (see below). RNA devices are also tunable, as point mutations in the RNA molecule affect its folding and/or strength of interaction with its partner RNA or DNA molecule, and hence its activity. Second, unlike TFs, regulatory RNA molecules do not require translation in order to function. Because of these advantages, synthetic RNA-based regulation is an expanding and exciting field. Some examples of synthetic RNA-based regulation are presented below.

Gene expression in mammalian cells can be knocked down by RNA interference (RNAi), complementary RNA sequences that target mRNA of interest for degradation in a sequence-specific manner [90]. RNAi molecules, such as short interfering RNAs (siRNAs) or microRNAs (miRNAs), can be chemically transfected into the cells or expressed from designated vectors to knock down a specific gene. RNAi may be used to build complex regulatory devices

through the combinatorial regulation of a common output transcript by multiple miRNAs. Benenson and colleagues used this property of miRNAs to build complex logic circuits in mammalian cells [23, 91, 92] (see Sect. 5 for details). In addition, in mammalian systems, specific 5′ intronic sequences and 3′ polyadenylation sites can be added to increase RNA stability and transgene translation [93]. Conversely, an mRNA can be destabilized with 3′ degradation tags, such as AU-rich elements (AREs) [94] to reduce transgene levels and decrease promoter leakiness.

Synthetic RNA-based devices also include small, *trans*-acting RNA molecules that base-pair with their target transcripts to either allow or inhibit transcriptional elongation or translation. Examples of such systems in *E. coli* include the riboregulator [95], the pT181 attenuator [96], and the RNA-IN-RNA-OUT system [97].

The riboregulator consists of two components: a *cis*-repressed RNA (crRNA) encoding the protein of interest, and a cognate small *trans*-activating RNA (taRNA) [95]. In absence of the taRNA, the 5′ untranslated region (UTR) of the crRNA forms a stem loop that blocks the RBS and prevents translation. When the taRNA base-pairs with its partner crRNA, the stem loop unfolds, allowing protein translation [95]. Conversely, the pT181 attenuator allows target gene expression only in the absence of the *trans*-acting RNA: the 5′ UTR of the target gene is engineered with an attenuator loop. In the presence of its partner antisense RNA, the attenuator loop folds into a hairpin that exposes a terminator site, preventing transcription of the downstream target gene [96]. In the absence of the antisense RNA, the target gene is transcribed [96]. While the riboregulator controls translation, and the attenuator regulates transcription, the RNA-IN-RNA-OUT system couples translational control to transcriptional elongation of a target gene [97]. The system consists of pairs of RNA molecules: an RNA-IN molecule and its cognate RNA-OUT molecule. The RNA-IN element is placed upstream of a sequence encoding a small regulatory peptide, *tnaC*, followed by the coding sequence of the target gene. RNA-OUT forms a complex with RNA-IN that blocks the translation of *tnaC*. In this system, the translation of *tnaC* is necessary to enable transcriptional elongation of the downstream target gene; hence, the presence of RNA-OUT blocks transcription of the gene coupled to RNA-IN [97].

Another widely used class of regulatory RNA molecules is the aptamer, an RNA molecule that specifically binds a ligand such as a small molecule or protein [40]. Aptamers may be used to build riboswitches, which regulate target gene expression in response to ligand binding. Naturally occurring riboswitches are found in bacterial RNAs encoding metabolic enzymes, where they regulate the enzyme's expression in response to the levels of the corresponding metabolite [98]. In synthetic circuits, riboswitches inserted into the 5′ UTR of a target gene modulate target gene expression by either permitting or blocking transcriptional elongation or translation initiation of the riboswitch-coupled transcript in response to a specific ligand [99, 100] (Fig. 1b; for a review, see Ref. [40]). Because of their usefulness, many studies have focused on screening for aptamers and riboswitches with desired activity. Aptamers that bind a ligand of interest may be selected *in vitro* from large pools of random nucleotides [101, 102]. Gallivan

and colleagues screened large libraries of riboswitches by coupling riboswitch activity to an easily observable readout, such as bacterial cell motility or fluorescence [103, 104]. Examples of ligands recognized by synthetic riboswitches include the small molecule theophylline [105], antibiotics [106], and dyes [107]. As an example of how aptamers can be used to control cell behavior, one study coupled the theophylline-responsive aptamer to the expression of CheZ, a protein necessary for chemotaxis in *E. coli*, resulting in *E. coli* cells that migrated toward a source of theophylline [108].

Aptamers may also be linked with catalytically active RNA molecules called *ribozymes* that can cleave or splice RNA. Such molecules, known as *aptazymes*, carry out RNA-modifying reactions in a ligand-responsive manner [109, 110]. In one example, the small molecule theophylline binds to its target aptamer and activates a ribozyme that cleaves the target mRNA, resulting in a downregulation of target gene expression in response to theophylline in *S. cerevisiae* cells [110]. A similar system was later implemented in mammalian cells to achieve theophylline-mediated mRNA degradation [111, 112]. Together, riboswitches, aptazymes, and *trans*-regulatory noncoding RNAs present an expansive toolbox for engineering fine-tuned gene expression in synthetic circuits.

2.6 Insulator Elements

In order for a synthetic circuit to function correctly, its component elements must be protected from unwanted interactions with other circuit components and with the host cell. Therefore, synthetic circuits may include insulators – DNA or RNA elements that insulate one part of a circuit from interference by an adjacent synthetic part or by the host's genome. For example, insulator sequences flanking a genetic circuit inserted into a mammalian chromosome help to prevent the circuit from becoming silenced due to local chromatin effects [113].

In another example, two RNA regulatory elements placed on one mRNA molecule may base-pair with each other and disrupt each other's function. To avoid this interference, the RNA regulatory elements may be separated physically through ribozyme-mediated cleavage [87]. A recently described alternative to ribozymes is the Csy4 endonuclease from the CRISPR system [114] (see Sect. 2.4), which cleaves RNAs at a 28-nucleotide recognition sequence that does not occur naturally in *E. coli*. Csy4 can efficiently cleave mRNAs engineered with the recognition sequence in *E. coli* and *S. cerevisiae*, and Csy4-mediated separation of transcript components such as the 5′ UTR and the coding region allowed expression levels to be protected from context effects in *E. coli* [114].

2.7 Post-Translational Regulation and Protein-Based Circuits

The behavior of transcriptional regulatory circuits depends in part on the stability of the proteins encoded by the circuit genes. Some applications, such as the oscillator (Sect. 3.1), require rapid degradation of the component TFs. In eukaryotes, protein lifetime can be tuned by fusing the protein to a tag that targets it for ubiquitin-dependent degradation [115]. In *E. coli*, the 11-amino acid *ssrA* tag targets proteins for rapid degradation by the ClpXP protease [116]. To achieve

inducible, fast degradation of synthetic proteins, Hasty and colleagues engineered an *S. cerevisiae* strain for expression of ClpXP from an IPTG-inducible promoter [41] (Fig. 1c). The addition of IPTG stimulates the production of ClpXP, which leads to a specific degradation of *ssrA*-tagged target proteins. The amount of ClpXP, and hence the degradation rate, can be tuned by the amount of IPTG added [41]. Moreover, specific tags have been developed that enable inducible protein degradation, in which protein degradation is inhibited by small molecules [117].

In addition, protein-based regulatory cascades may be designed and modeled after intracellular signal transduction pathways found in eukaryotic cells [118]. Protein-based signaling pathways have several advantages over transcription-based circuits. Protein-based signaling occurs at much faster timescales than transcription; moreover, protein-based signal transduction relies on processes (such as protein phosphorylation or conformational change) that require little of the cell's energy and resources. However, to design protein-based synthetic devices presents unique challenges, mainly because it is currently extremely difficult to predict a protein's function and activity from its amino acid sequence. Instead of rationally designing proteins *de novo*, the construction of synthetic post-translational regulation relies on repurposing catalytic and regulatory domains from naturally occurring signaling proteins, and wiring them together in novel ways. Key examples include rewiring the pheromone signaling response in *S. cerevisiae* [42] (Fig. 1d), as well as using bacterial proteins to attenuate signaling pathways in yeast and in human T cells, with possible applications for T-cell-based therapy [119].

3 Dynamical Circuits

The building blocks described above have been used to construct synthetic gene circuits of varying complexities and purposes. The first synthetic gene circuits that were constructed were not intended for practical applications, but were simple dynamical networks intended to demonstrate the proof-of-principle ability to engineer gene circuits with desired behaviors. Early examples of dynamical circuits were described in 2000 [8, 9], and have formed the basis (either conceptually or practically) for much work on synthetic circuits since then, whether applied or not. These two circuits were the oscillator and the switch.

3.1 Oscillators

Oscillators are common components in electronic devices [120]. An oscillator stably interchanges between two states and can be characterized by its amplitude and period. The first synthetic gene oscillator circuit was the *E. coli* "repressilator" [8], a ring of three transcriptional regulators, LacI, TetR, and CI, each repressing the next one in the ring (Fig. 2a). As LacI represses the transcription of *tetR*, it relieves the repression by TetR on the *cI* gene, allowing CI levels to rise and repress *lacI*; the now de-repressed *tetR* gene product begins to accumulate until it can repress *cI*, and the cycle continues. Studies on the repressilator sparked almost a decade of focus on synthetic gene oscillators (see Ref. [121] for an extensive analysis). Studies on oscillators illustrate the importance of circuit topology and parameters on overall circuit function, and provide an example of the "design–build–test" cycle of synthetic circuit engineering. Following the

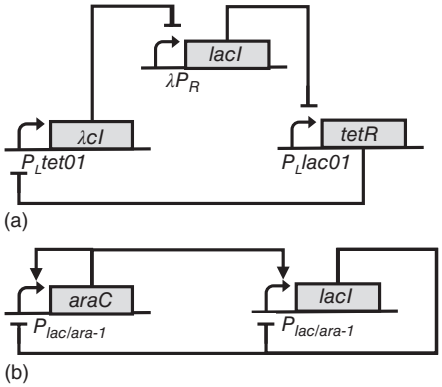

Fig. 2 Synthetic gene oscillators. (a) The repressilator consists of three genes in a ring, each repressing transcription from the next one in the chain [8]. Gene and promoter names are given; (b) The robust oscillator constructed by Stricker *et al.* uses a combination of positive and negative feedback [122]. In both circuits the arrows represent transcriptional activation, while flat-ended arrows represent transcriptional repression. Modified from Ref. [122].

initial repressilator, a number of improved versions were designed and implemented.

Despite being a significant breakthrough, the repressilator was not a robust circuit as it only functioned in about 40% of cells. It was not until 2008 that a highly robust oscillator circuit was constructed [122]; this oscillator had a different topology from the repressilator, comprising one gene that activates itself and another gene, this second gene feeding back to and repressing the first gene (Fig. 2b). This design is known as an *amplified negative feedback oscillator*, where ''amplified'' refers to the first gene's positive feedback on itself. A number of other oscillators have been constructed, the majority within the bacterium *E. coli* [123, 124], and one in *Salmonella typhimurium* [125]. For example, one study implemented a metabolic oscillator using fluctuations in metabolite pools [123].

Oscillators have also been successfully introduced into mammalian cells. In a negative feedback loop-based oscillator, an intron placed upstream of a repressor protein coding region increases oscillatory period length with the size of the intron, due to increased transcript length [126]. In another example, a tunable oscillator was constructed by combining a feedforward loop and a time-delayed negative feedback loop based on an autoregulated sense–antisense transcription control [127]. A subsequent study produced a low-frequency oscillator, in which a time-delayed negative feedback loop was based on a short hairpin RNA (shRNA) encoded in the intron of a self-regulated transactivator [128]. Finally, synthetic–natural hybrid oscillators can be constructed in human cells based on the structure of natural networks, such as the p53 pathway [129].

Now that oscillators can be constructed routinely, the challenge is to integrate them into larger synthetic circuits. A potential application is to use oscillators as timer circuits, keeping time for the rest of a synthetic circuit. Oscillators may also be used in frequency multiplier circuits, allowing different parts of a larger circuit to be kept at different timings [130]. Attempts to connect oscillators to downstream circuits have highlighted a common issue that is known to electrical engineering when connecting up different circuits: *retroactivity*, which occurs when a downstream component of a circuit affects its upstream components [131, 132]. In genetic oscillators, retroactivity occurs because some of the protein used in the oscillator itself has to be used to drive the downstream circuit by binding to the downstream gene promoters. This

binding sequesters the oscillator protein, affecting the oscillator dynamics. One approach to minimizing retroactivity is to use an amplifier circuit as an intermediate between the oscillator and the downstream circuit. The amplifier requires only a small amount of protein from the oscillator, minimizing the effect on the oscillator, but is strongly expressed, giving a large output sufficient to drive the downstream circuit. This approach has been successfully demonstrated in an *in vitro* synthetic oscillator based on hybridization of synthetic DNA and RNA oligonucleotides (see Ref. [133] for details).

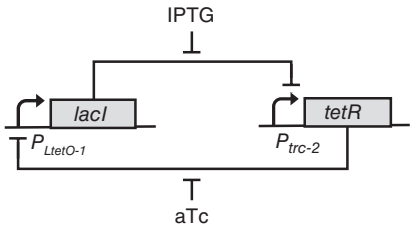

Fig. 3 The toggle switch. The original synthetic toggle switch in *E. coli* consists of two repressors, *lacI* and *tetR*, which repress each other's transcription [9]. The small molecules aTc and IPTG bind to TetR and LacI proteins, respectively, preventing them from repressing transcription and therefore allowing expression of *lacI* and *tetR*, respectively. Flat-headed arrows depict transcriptional repression. Adapted from Ref. [9].

3.2 Toggle Switches

Along with the oscillator, another example of a simple functioning device is a toggle switch: a circuit that can switch between two stable states, ON and OFF, similar to the light switch of a lamp. As a result, a switch possesses a form of memory, which is vital for cellular computation (see Sect. 4). The genetic switch, commonly called a *toggle switch*, consists of two transcriptional repressors, each repressing the other (Fig. 3). Collins and colleagues described a functional toggle switch in a living *E. coli* cell [9]; the switch uses two small molecule-responsive transcriptional repressors – TetR, which binds aTc, and LacI, which binds IPTG (see Sect. 2.2). The addition of aTc inactivates TetR, switching the system into a state with high expression of LacI, which then maintains a low concentration of TetR. Conversely, addition of IPTG inactivates LacI, switching the system into a high-TetR state, which then maintains a low concentration of LacI. Another version of the toggle switch uses a heat pulse as a means of switching off a temperature-sensitive transcription repressor [9].

A mammalian toggle switch was also developed based on two transcriptional repressors, enabling it to switch between states according to two antibiotic inputs [134]. Subsequently, an RNAi-based toggle switch was designed for the tight regulation of gene expression in mammalian cells that switch between states according to small-molecule inputs [135]. The switch could be used to tightly control the expression of potentially useful proteins, such as the toxic alpha chain of diphtheria toxin and the pro-apoptotic protein BAX (BCL-2-associated X protein) that can kill cancer cells. Recently, a light-responsive toggle switch was developed, in which gene expression is induced by red light and silenced by far-red light [136].

As is the case with the oscillator, the function of the toggle switch depends on the relative expression levels of its component transcription repressors – the mutual repression between the two genes must be

approximately balanced. By unbalancing the relative levels of the two repressors, Ellis *et al*. were able to convert the *E. coli* toggle switch into a genetic timer [137]. In this way, the authors changed the dynamics of the switch from bistable to monostable, so that the circuit would return to its initial state after perturbation. In this implementation, the toggle switch was set up to return to a stable state with low LacI expression. Induction with aTc moved the system into a high-LacI, low-TetR state. Upon the removal of aTc, the system eventually returned to low LacI and high TetR conditions. The time needed to reset the system confers the circuit's timer action, which can be controlled by changing the strengths of the various promoters [137].

A further advance in switches was made with the construction of a push-on-off switch [138]. Whereas, the toggle switch requires two inputs, the push-on-off switch switches repeatedly back and forth between two states using just one input. This circuit was larger and more complex than the original toggle switch (see Ref. [138] for details), and was not particularly robust; the fraction of cells switching decreased quickly as the number of rounds of switching increased, in part because the input used – UV light – caused cell lethality. The authors proposed ways to improve future implementations of the circuit, including using an input that would be less harmful to the host cell [138].

Like oscillators, toggle switches can also be coupled to cell behaviors of interest. For example, Kobayashi *et al*. built a toggle switch circuit in *E. coli* to induce biofilm formation in response to DNA damage [139]. The toggle switch consisted of the CI and LacI TFs mutually repressing each other. This circuit starts out in a low-LacI, high-CI state. A pulse of DNA-damaging radiation then triggers expression of the native RecA protease, which cleaves CI and flips the toggle switch into low-CI, high-LacI state, which in turn promotes expression of a gene required for biofilm formation [139].

In a recent study, a synthetic toggle switch was used to address a fundamental question in cell biology: How can cells in an isogenic population achieve distinct cell fates when starting from the same state [140]? The authors implemented a toggle switch in *S. cerevisiae* that can exist in two states: the high-LacI/low-TetR state, which expresses green fluorescent protein (GFP) but not mCherry; and the low-LacI/high-TetR state, which expresses mCherry but not GFP. Addition of the TetR inhibitor aTc switches the cells into the high-LacI, GFP-positive state. The system was ''initialized'' by growing the cells in glucose, which blocked expression of all synthetic proteins. The cells were then transferred to galactose media to permit protein expression, and their fate (high versus low GFP) was observed in the presence of varying amounts of aTc. In the presence of an intermediate aTc concentration, the isogenic cell population eventually reached a bimodal distribution, with approximately equal numbers of cells becoming GFP-positive and GFP-negative [140]. In contrast, when given a high or low dose of aTc, all cells reached a high-GFP or low-GFP state, respectively. Moreover, the authors showed that the intermediate aTc concentration that leads to a bimodal distribution of cell fates depends on the expression levels of LacI and TetR: for example, a cell line engineered to express a lower amount of TetR relative to LacI will need less aTc to reach a bimodal distribution. The experimental evidence and accompanying mathematical model indicate that isogenic cells can stochastically reach distinct cell fates when

exposed to a signal whose concentration is in a critical intermediate range, which depends in turn on the expression levels of the cell's regulators that respond to the signal [140]. In summary, the authors used a simple synthetic circuit in order to gain insight into a complex biological phenomenon.

3.3 Gene Cascades

In addition to the repressilator and toggle switch, synthetic biologists have built genetic cascades: systems in which an upstream gene product regulates a downstream gene, which in turn regulates a gene further downstream, and so on. Weiss and colleagues [141] constructed synthetic cascades of various lengths in *E. coli* (Fig. 4) in order to understand how the characteristics of the cascade relate to its length (i.e., the number of genes in the cascade). Longer cascades were shown to have sharper switching between ON and OFF states, and to display noisier dynamics. As predicted, the response time increased with the length of the cascade. This feature allowed the longer cascade to act as a low-pass filter, with the output less affected by rapid fluctuations in the input than fluctuations persisting for a longer time. However, this filtering ability came at the expense of synchrony: the longer the cascade, the more variability was observed in response time across the cell population. The authors pointed out that genetic cascades are common in Nature, but that if the cascade regulates a process that requires the cells to act in concert (such as development of a multicellular organism), then additional regulatory mechanisms must exist to ensure synchrony [141].

3.4 Bandpass Filters

In electronics, bandpass filters are made to block the transmission of any wavelength under and above a specific range, thus allowing only a specific range of wavelength with a defined minimum and maximum

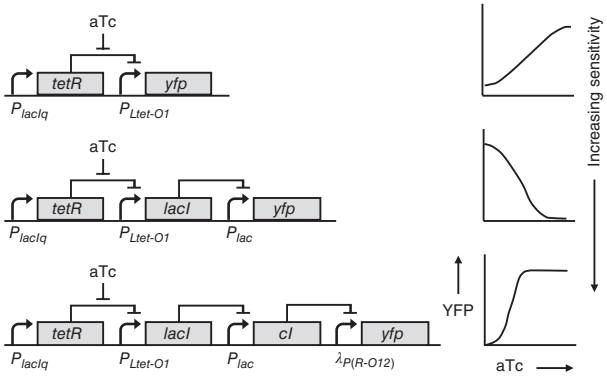

Fig. 4 The properties of synthetic gene cascades vary with cascade length. Cascades of two, three, and four genes were constructed [141]. aTc binds to TetR protein, preventing it from repressing transcription from a downstream promoter. The graphs on the right show the steady-state relationship between the concentration of aTc (input) and yellow fluorescent protein (YFP) fluorescence (output). As the length of the cascade increases, this relationship becomes more sensitive, with sharper switching between ON and OFF states [141]. Modified from Ref. [141].

to pass [142]. In biology, a bandpass filter can be used to limit the response of a gene network to predefined biological input levels, ignoring inputs that are too weak or too strong.

A tunable bandpass filter was implemented in *E. coli* using a feedforward loop in which a β-lactamase gene (*bla*) and a tetracycline resistance gene (*tetC*) are linked by mutually repressive interactions [32]. The Bla enzyme allows the bacteria to survive in ampicillin (Amp) by hydrolyzing Amp. A minimum threshold level of Bla is required for the cell to survive in the presence of Amp, but if Bla accumulates above the maximum threshold level, this leads to a repression of the *tetC* gene and hence makes the cell sensitive to tetracycline (Tet). As a result, the circuit acts as a bandpass filter when the cells are grown in the presence of both Amp and Tet: only cells with a specific range of circuit activity, assayed by a GFP reporter, will survive. An interesting feature of the circuit is the control of the *bla* gene by an IPTG-inducible promoter, which makes the circuit tunable: varying the IPTG concentration in the media changes the properties of the bandpass filter (the concentration of Amp and Tet that allows the cell to survive) [32]. A bandpass filter was also built in mammalian cells. Through a combination of interconnected transcriptional activators and repressors, the cells were programmed to express a reporter gene only in the presence of intermediate input concentrations [143].

4 Memory Devices

Many synthetic circuits respond to specific inputs quickly and reversibly: the cell performs a desired action in the presence of input such as a small molecule or a specific wavelength of light, and then, once the input is withdrawn, the cell returns to its ''ground'' state. The kinetics of the circuit's response to the input are important for its function, and many applications require the circuit to return to the pre-input state promptly.

In contrast, some applications require the cell to maintain memory of a transient stimulus, even over long time periods and multiple generations. One potential use of a memory circuit is to record the cell's history of intrinsic or extrinsic events, such as exposure to toxic chemicals or DNA damage, that are relevant in environmental remediation and medical diagnostics. A counter enables the cell to remember not only prior exposure to a stimulus, but also the number of times it was exposed. Memory circuits may enable engineered microbes to respond to complex and uncertain environments, based not only on current input but also on memory of past events; this ability would allow more complex cell responses than those permitted by simple digital logic (see Sect. 5). Potential uses include bioremediation, support for crops growing in marginal soil, or disease treatment [144]. Moreover, during the development of a complex multicellular organism, each cell must at some point commit to a specific cell fate, and its progeny must maintain memory of the cell fate choice. Synthetic memory devices may mimic the complex cellular logic implemented during differentiation; for example, a memory device may produce distinct outputs depending on the sequence of inputs (A then B versus B then A). These memory circuits may be useful in studying or programming cellular differentiation for applications such as tissue engineering.

Synthetic memory can be divided into volatile and nonvolatile [145]. Volatile memory requires the cell to maintain the memory of the past event actively, whereas nonvolatile memory is passively maintained by the cell following the initial stimulus. These memory systems can be analogized to dynamic and static memory in electronic systems, respectively. Many of the synthetic memory devices, such as the autoregulatory feedback loop described below, resemble those that occur naturally in cells and function extensively in processes such as cell development [146, 147]. In mammalian cells, naturally occurring long-term memory also relies heavily on the chromatin state, which may present another opportunity for implementing synthetic memory devices [148]. Currently, an incomplete knowledge of the mechanisms and outcomes of specific chromatin modifications has limited the ability to design chromatin-based synthetic memory devices. However, synthetic chromatin-modifying factors have been described [149], and synthetic zinc finger- and TALE-based DNA-binding domains have allowed chromatin-modifying proteins to be targeted to specific loci in living cells [39, 80] (Sect. 2.4). A greater understanding of chromatin modifications may enable a more sophisticated and reliable use of chromatin-based synthetic circuits in the future.

4.1 Volatile Memory

A volatile memory device requires active maintenance of the memory state by the cell (e.g., through transcriptional regulation). The toggle switch (see Sect. 3.2), in which two genes, each responsive to a distinct input, repress each other's expression, is an example of volatile memory: the cell remembers the last input it received, even after the input is withdrawn, but maintenance of this memory requires active gene expression [9, 134].

Volatile memory has also been implemented through autoregulatory feedback loops, which consist of two sTFs: a sensor TF and a self-activating ''loop'' TF. A transient stimulus activates the sensor TF, which in turn initiates expression of the loop TF. Once the loop TF accumulates above a threshold level, it maintains its own expression in a positive autoregulatory feedback loop even after the sensor TF becomes inactivated [18, 150]. In an early example, *S. cerevisiae* cells were programmed to turn on long-term yellow fluorescent protein (YFP) expression in response to a transient galactose input [150]. In a subsequent study, mammalian cells were programmed to remember past exposure to hypoxia and DNA damage, both of which are linked with cancer [18].

Another circuit design exhibits a different form of memory: the ability to count three pulses of a defined small-molecule input [151]. In this circuit, expression of the final output (GFP) depends on a series of orthogonal phage-derived RNAPs that are under the control of a riboregulator (see Sect. 2.5): the RNAP-encoding transcripts cannot be translated, due to *cis*-repressive RNA structures present in the transcripts. Each pulse of the inducer triggers the expression of a small *trans*-acting RNA, which relieves the RNA-based repression and allows each RNAP transcript to be translated. After three pulses of the inducer, the cascade is complete and the GFP reporter is highly expressed [151]. The device requires the pulses to be spaced closely together, to prevent each RNAP from being degraded or diluted by cell division before the next RNAP in the cascade can be expressed.

4.2 Nonvolatile Memory

In contrast to volatile memory, nonvolatile memory does not require continuous action by the host cell; rather, the memory device retains its state passively after the initial switching event. Nonvolatile memory devices have been implemented using site-specific DNA recombinases that can insert, excise, and invert DNA fragments at specific DNA sequence motifs. Advantages of nonvolatile memory devices include a lower metabolic burden on the cell, because the cell does not expend energy to maintain the memory state, as well as the ability to construct a multistate circuit from relatively few parts by arranging the recognition sites for two or more orthogonal DNA recombinases. The number of possible states of the system grows exponentially with the number of orthogonal recombinases available [144].

Notably, unlike volatile memory, nonvolatile memory based on DNA recombinases persists after the removal of the memory device (the rearranged DNA fragment) from the host cell. This has led to the intriguing idea that such memory devices could be shared among cells, via the transformation of a ''naïve'' cell with DNA from a lysed memory cell [144], leading in turn to the possibility of complex multicellular computation.

Examples of synthetic nonvolatile memory include the use of bacterial invertases that catalyze inversion of a fragment of DNA flanked by specific inverted repeat sequences [144, 152]. In one example, induction of the *E. coli* FimE invertase leads to an irreversible flipping of the promoter of the *gfp* reporter gene from the OFF state to the ON state [152]. In a more complex follow-up study, the use of two orthogonal invertases (Fim from *E. coli* and Hin from *Salmonella*) allows for the programming of devices with many possible states, including devices that can produce different output depending on the history of the system: Fim followed by Hin, versus Hin then Fim (Fig. 5a) [144]. While these studies presented an exciting proof-of-principle, the resulting circuits did not behave entirely as expected, due partly to the very low rate of inversion by Fim [144].

Another early example of a recombinase-based synthetic memory device is the DNA invertase cascade in *E. coli* that can count to three: reporter GFP expression becomes active only after three pulses of an inducer molecule [151]. In this system, each pulse of the small molecule input drives expression of a recombinase, which in turn catalyzes a DNA inversion that prepares the circuit to respond to the next inducer pulse. One version of the circuit counts three pulses of the same small molecule, arabinose. Another version respond to pulses of three different inducers given in a specified order: for example, the circuit expresses GFP if aTc, arabinose, and IPTG are supplied in that order, but not if the order of the stimuli is rearranged [151]. This system is useful for modeling processes such as development, where the timing of the stimuli matters in addition to the identities of the stimuli.

Other studies in this area have utilized phage-derived serine recombinases. Originally used in bacteria, codon-optimized recombinases also function in eukaryotic cells. These enzymes catalyze recombination between two recognition sites, termed *attP* and *attB* (attachment phage and attachment bacterium, respectively), resulting in the formation of *attL* and *attR* sites (Fig. 5b). Because the recombinase does not recognize *attL* and *attR* sites, the reaction is irreversible unless an excisionase is

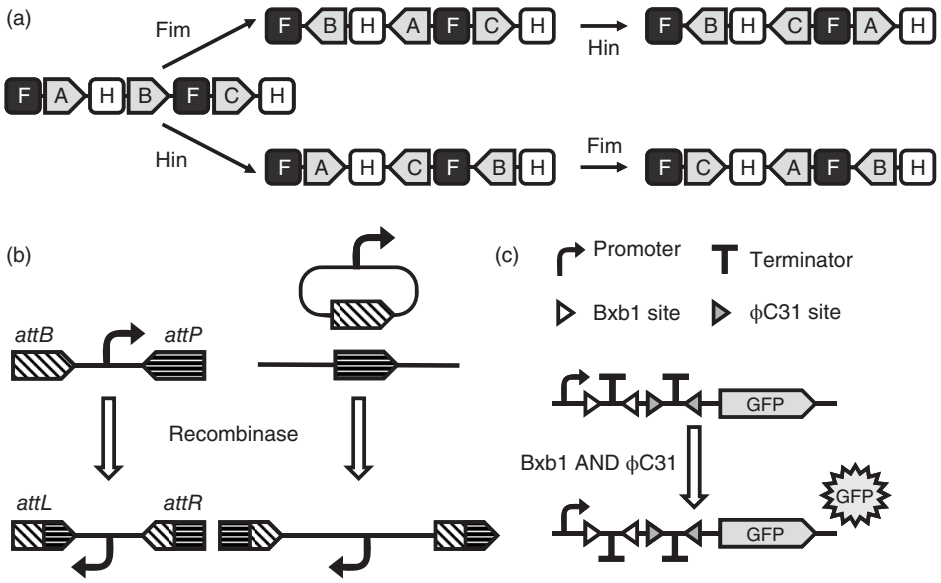

Fig. 5 Synthetic nonvolatile memory devices. (a) The bacterial invertase-based memory device uses two orthogonal enzymes, Fim and Hin, which mediate DNA inversion between sites marked "F" and "H," respectively. Note that the final state of the system depends on the order of inputs: Fim followed by Hin versus Hin followed by Fim [144]; (b) Phage-derived serine recombinases catalyze DNA inversion (left) or integration/excision (right) between specific recognition sites, termed *attB* and *attP*. The reaction produces *attL* and *attR* sites, which are not recognized by the recombinase, making the reaction irreversible unless a cognate excisionase is added; (c) An example of a two-input digital logic gate implemented with two orthogonal serine recombinases, Bxb1 and ϕC31 [153]. In this AND gate, two transcriptional terminators prevent expression of the GFP reporter from the upstream promoter. When both recombinases are present, the two terminators are switched to the "off" position, and GFP expression can proceed. Panel (a) modified from Ref. [144]; panel (c) modified from Ref. [153].

added (see below). Depending on the location and orientation of the starting *attB* and *attP* sites with respect to each other, the reaction results in excision, integration, or inversion ("flipping") of a DNA fragment (Fig. 5b). Site-specific recombinases from multiple phages have been described, including TP901, Bxb1, and ϕC31 [154]. Notably, these recombinases are orthogonal, with each recognizing a specific and unique pair of *attP* and *attB* sites, a property that allows the use of multiple recombinases in a single cell.

A recent study demonstrated the construction of all 16 two-input Boolean logic gates (see Sect. 5) in *E. coli* using two orthogonal site-specific recombinases, Bxb1 and ϕC31 [153]. In this study various combinations of promoters, transcriptional terminators and reporter coding regions flanked by recombinase target sites were used to achieve the desired logic function. For example, AND logic was implemented by inserting two transcriptional terminators, one flanked by Bxb1 sites and the other by ϕC31 sites, between a promoter and a GFP-coding region, such that the two terminators were flipped to the OFF orientation and GFP was expressed only in the presence

of both recombinases (Fig. 5c). The expression of the two recombinases was coupled to two different small-molecule inputs, so that Boolean logic computations were performed upon addition of the specified input molecules. As with the previous examples of recombinase-based circuits, the circuits passively maintained their state upon removal of the stimulus.

Although a serine integrase alone catalyzes an irreversible reaction ($attB + attP \rightarrow attL + attR$), the combination of the integrase with a cognate excisionase can catalyze the reverse reaction and restore the *attB* and *attP* sites. A recent study harnessed this phenomenon to build a reversible memory module in *E. coli* [155]. In this system, expression of the Bxb1 integrase alone leads to red fluorescent protein (RFP) reporter expression; coexpressing the Bxb1 integrase with its partner excisionase inverts the orientation of the reporter gene promoter, resulting in GFP expression [155]. In theory, the system can ''flip'' repeatedly between the GFP-on state and the RFP-on state through repeated induction of integrase alone or integrase with excisionase. However, functioning of the system requires careful adjustment of the integrase:excisionase ratio [155].

5 Boolean Logic and Digital Circuits

Most of the synthetic gene circuits that have been implemented so far have benefited from digital logic design, in which cellular networks are thought of as assemblies of digital logic gates. A digital logic gate is a device that carries out a Boolean operation and produces a Boolean output based on the different combinations of inputs it receives. Both the inputs and the output of a logic gate are in the form of TRUE and FALSE, often represented as 1 and 0, respectively. Examples of basic Boolean operations include AND (output is TRUE only if all inputs are TRUE), NAND (output is TRUE unless all inputs are TRUE – the negation of AND), OR (output is TRUE if at least one input is TRUE), and NOR (output is true if none of the inputs is TRUE – the negation of OR) (Fig. 6). Most digital logic gates used in synthetic biology have been two-input gates, of which there are 16 types. Interconnecting such two-input logic gates so that the output of some gates acts as input for others can be used to design more complex logic circuits.

The domain of digital logic computation in living cells is modeled after engineering electronic circuits, with the goal of programming cells similarly to programming a digital computer, only with biological inputs and outputs rather than electronic ones. Instead of physical wires, molecules such as RNA and protein connect logic gates inside the cells. Such circuits could be implemented for many purposes, including diagnostics and therapeutics, in which the cell is designed to perform a specific behavior in response to specific stimuli, such as ''if the inputs match the profile of a cancer cell, kill the cell'' [22, 23, 156–158]. Moreover, since biological inputs can be converted to electric ones and vice versa [136, 159–161], cells could potentially be integrated as biological computational units for electronic computers.

Compared to the assembly of their silicon-based counterparts, the construction of complex biological logic circuits by layering elementary logic gates has proven to be extremely challenging. Over the past decade, many different designs with various functions have been implemented in both prokaryotic and eukaryotic cells, but the level of complexity of these designs

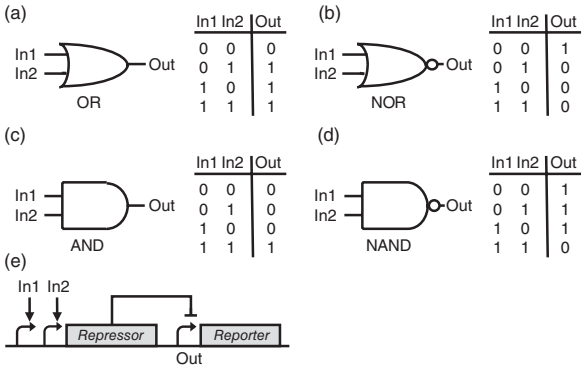

Fig. 6 Digital logic gates. A few examples of two-input logic gates as well as the truth table that each one encodes are shown. (a) OR gate; (b) NOR gate; (c) AND gate; (d) NAND gate. A truth table summarizes the outputs to each possible combination of inputs of a logic gate. The logic gates are drawn according to the conventions of electrical engineering. The bubble in (b) and (d) symbolizes negation; (e) Schematic representation of a genetic NOR gate: the presence of either or both of the two inducers activates the expression of the repressor protein, which in turn represses the reporter output. Hence, the output is expressed only when neither of the inducers is present, as per the truth table shown in panel (b). In1, input 1; In2, input 2; Out, output.

has not improved significantly [162]. This is in part due to unwanted crosstalk between components of synthetic circuits (Sect. 2.3), the propagation and amplification of noise through the circuit (Sect. 8.3), and the metabolic burden that accompanies the expression of exogenous genes in host cells (Sect. 8.2). In this section, a few hallmark examples of synthetic digital logic circuits that have been implemented are briefly provided.

An early example of a digital logic circuit used chimeric promoters that bind small-molecule-responsive transcriptional activators and repressors. Depending on the combination of activators and repressors used, the system gave rise to different logic gates, including AND, NAND, and NIMPLY (output is TRUE only when one specific input is TRUE) gates [163]. Subsequent studies employed split inteins which, when fused to separate proteins, would mediate fusion of the two proteins into one. Hence, split inteins would allow the reconstitution of a functional TF that can activate or repress an output promoter, forming an AND or NAND logic gate, respectively. Split inteins fused to sequence-specific ZF-TFs (see Sect. 2.4) were used to build a variety of logic gates in mammalian cells [70]. Recently, the split intein strategy was used to build AND gates in mouse stem cells [164], suggesting the possibility of future applications in programming stem cell fate for research and therapy.

Other digital logic circuits combined transcriptional and post-transcriptional regulation. For example, Arkin and colleagues [165] constructed a two-input AND gate in *E. coli*. The first input activates transcription of a gene encoding T7 RNAP, but the protein cannot be translated due to the presence of two premature amber stop codons. The second input turns on expression of an amber suppressor tRNA, so that the presence of both inputs allows expression of T7 RNAP protein and subsequent transcription of the reporter gene from a T7 promoter [165]. The authors

linked the AND gate to the expression of Invasin in bacteria, such that the bacteria invaded human cancer cells *in vitro* only in the presence of two user-defined inputs [165, 166]. This circuit represents a proof-of-principle strategy that may be used for cancer therapy in the future.

Digital logic circuits may also be implemented in mammalian cells using miRNAs, as shown by Benenson and colleagues, who built computational devices in mammalian cells by targeting multiple miRNAs to the same output transcript [91, 92]. Expression of the miRNAs may be coupled to the presence or absence of user-defined transcriptional activators or repressors, which act as inputs to the circuit [92]. For example, if a transcript that encodes a fluorescent protein output has recognition sequences for two miRNAs – miR-a, which is repressed by TF-A, and miR-b, which is activated by TF-B – then the resulting circuit will produce fluorescent output only if A is present and B is absent, implementing the digital logic function A AND NOT B. More complex circuits may be built by combining multiple miRNA-responsive output transcripts in one cell [91, 92].

The potential for practical applications of miRNA-based logic was demonstrated by a circuit that induces cell death specifically in the HeLa cancer cell line [23] (Fig. 7). The circuit accepts as inputs the levels of two user-defined sets of miRNAs: those expected to be highly expressed and those

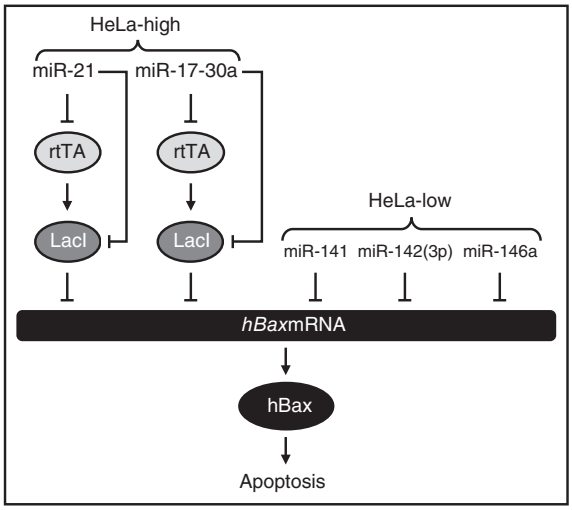

Fig. 7 Logic computation in mammalian cells: the miRNA classifier circuit. The HeLa cancer cell classifier causes production of the pro-apoptotic protein hBax and hence the death of the host cell if, and only if, all ''HeLa-high'' miRs are highly expressed and none of the ''HeLa-low'' miRs is expressed above a threshold level. The ''HeLa-high'' miRs regulate an inverter module: the miRs prevent expression of the rtTA transcription activator, which in turn activates expression of the LacI transcription repressor (the miRs also target the lacI transcript to improve the signal-to-noise ratio). Hence, in HeLa cells, the ''HeLa-high'' miRs prevent expression of the LacI protein, permitting transcription of hBax mRNA, which has sequences complementary to ''HeLa-low'' miRs in its 3′ UTR. In a healthy cell, high levels of one or more of the ''HeLa-low'' miRs block hBax expression, whereas in HeLa cells hBax translation can proceed. As a result, only HeLa cancer cells undergo apoptosis in the presence of the classifier circuit. Adapted from Ref. [23].

expected to be absent from HeLa cells (''HeLa-high'' and ''HeLa-low'' miRNAs, respectively). The output of the circuit is the pro-apoptotic protein BAX, which triggers cell death. The ''HeLa-high'' miRNAs prevent expression of the LacI transcriptional repressor, which in turn represses the BAX-encoding gene. Hence, BAX protein expression is possible only when the ''HeLa-high'' miRNAs are present above a threshold level. In parallel, ''HeLa-low'' miRNAs downregulate the BAX transcript via their recognition sites in the BAX transcript 3′ UTR. Consequently, the circuit leads to BAX expression if, and only if, the host cell matches the HeLa cell profile – that is, all ''HeLa-high'' miRNAs are above a threshold level and all ''HeLa-low'' miRNAs are below a threshold level [23]. The circuit triggers significantly higher levels of apoptosis in HeLa cells compared to control human embryonic kidney (HEK) cells, indicating its potential for future cancer therapy [23]. Depending on the miRNA inputs used, the circuit could be designed to target any cell type that has a unique miRNA profile.

A subsequent study in mammalian cells used two small-molecule inputs – erythromycin and phloretin – to drive the expression of two transgenes: one that encodes the RNA-binding proteins MS2 or L7Ae; and one that contains the fluorescent reporter coding region fused to a RNA box sequence that specifically binds MS2 or L7Ae [167]. When bound to their cognate RNA boxes, MS2 and L7Ae block translation of the reporter transcript. This enables construction of NIMPLY gates, in which the fluorescent protein is expressed only if the input that activates its promoter is on, and the input that activates expression of the RNA-binding protein is off. By combining two NIMPLY gates in one cell, the technically challenging task was accomplished of constructing a two-input XOR gate, which outputs TRUE only if exactly one input is TRUE [167].

While most digital logic gates involve transcriptional regulation, RNA-based, post-transcriptional mechanisms have also been used [87] (see Sect. 2.5). In a recent study, digital logic gates were demonstrated in mammalian cells based on DNAzymes, which are synthetic DNA molecules that bind to and cleave a transcript in a sequence-specific manner [168]. The DNAzymes include inhibitory stem-loop structures, which block their catalytic activity unless relieved by binding to a specific miRNA. The use of DNAzymes allowed the construction of logic gates with specific miRNAs as inputs, and translation of the target mRNA as output. This method could potentially be used to detect miRNA profiles associated with cancer [168].

The above-described studies demonstrated the potential of using digital logic circuits to customize cellular signaling and regulatory networks to achieve various useful applications. However, these logic circuits were relatively simple, and most of them had single-layer gates. In an effort to build a complex layered logic circuit in *E. coli*, Moon *et al.* layered individual AND gates to integrate signals from four different inputs [169]. In their design, AND gates accept two promoter inputs and control one promoter output. Each gate is composed of a TF that needs a second chaperone protein in order to activate the output promoter. The authors applied directed evolution and part mining (see Sect. 2.3) to minimize crosstalk between the gate components, allowing the gates to be layered in a single cell. The result was a four-input AND gate, the most complicated circuit implemented in single cells to date [169].

TF-based logic circuits are limited by the shortage of truly orthogonal parts, as well as the metabolic burden they impose on the host cell; for example, the four-input AND gate requires 11 regulatory proteins [169]. One alternative approach would be to use recombinase-based circuits, which combine digital logic and memory [153, 170] (see Sect. 4.2). The memory module can be used to design robust and multilayered synthetic circuits, and the one-time inversion of a piece of DNA by a recombinase places less metabolic load on the cell than does the continued expression of a TF. Alternatively, RNA-based regulatory devices (see Sect. 2.5) can be composed into logic gates and cascades that place a lower metabolic burden on the cell than does the expression of heterologous regulatory proteins. For example, a four-input NOR gate based on the RNA-IN-RNA-OUT system in *E. coli* [97] achieved robust digital behavior while requiring a fraction of the resources of the protein-based four-input AND circuit [169] (see Sect. 2.5 for details).

6 Analog Circuits

As described above (Sect. 5), the majority of synthetic gene circuits built thus far were designed to perform in the digital domain. However, digital computation requires the composition of a large number of orthogonal devices together, each performing a simple binary computation, to achieve more complex functionalities. Although this is possible in the world of semiconductors due to the ability to assemble billions of transistors on a single substrate and to wire them together in a precise fashion, it is challenging to do in biological systems due to the paucity of available parts as well as resource limitations such as energy and space.

An alternative strategy would be to use analog computation, which leverages the inherent physics of a system to calculate mathematical functions and can thus achieve a greater computational complexity with fewer resources. Synthetic biologists have designed and fabricated circuits that function in the analog domain, where a graded input is converted into graded levels of output. The digital paradigm can be considered as a special case of graded analog functions where values below a given threshold are defined as ''0'' and values above that threshold are classified as ''1'' (Fig. 8a). A key difference between digital and analog circuits is the way in which they can be composed together in order to construct higher-order functions. For example, an analog adder can be achieved by simply combining two parallel circuits that each have different input molecules but generate the same output molecule [171]. However, a digital adder cannot function correctly using the same principle; for example, adding two ''1'' binary numbers together requires another stage to hold the new bit ''Carry out'' (''10'') (see Ref. [172] for an example of an adder implemented with digital logic in yeast).

The main advantage of analog computation in synthetic biology is the ability to implement complex mathematical functions using a small number of synthetic components with high efficiency. For example, in theory a circuit can be built that computes an integral function by measuring the accumulation of a protein over time, or a differential function by measuring the consumption of a protein over time. Moreover, analog circuits are of interest to synthetic biology because many cellular processes do not rely on the all-or-none responses found in digital circuits; rather,

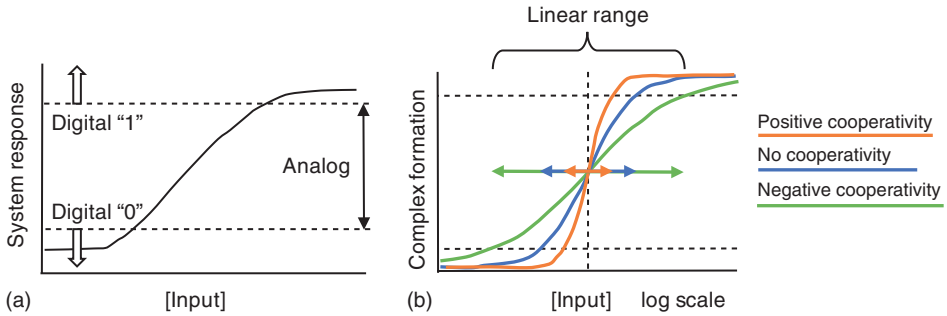

Fig. 8 Schematic representation of the difference between analog and digital circuits. (a) Abstraction of analog and digital circuits. A digital circuit recognizes only the difference between values above (1) and below (0) specific threshold levels. An analog circuit recognizes differences between graded levels of input in the linear portion of the input–output function; (b) Implementation of Weber's law and linear range in basic biochemical reactions. Negative cooperativity expands the linear range of the reaction. Modified from Ref. [171].

the cell responds in a graded fashion to changes in input concentration [173]. For example, increasing the levels of arabinose in the media activates progressively higher levels of expression of arabinose transporters and metabolic enzymes in *E. coli* [174]. Hence, analog circuits are suitable for programming cell behaviors for possible practical applications.

Living cells sense their environment using sensory systems, many of which follow Weber's law, to integrate environmental information such as light, sound, and chemotaxis [175]. Weber's law represents an analog rather than digital behavior and defines situations where the fold-change between signal levels and its background is a constant, thus enabling fold-change detection as opposed to absolute level detection [173]. Weber's law also applies to molecular signaling networks and biochemical reactions. Figure 8b shows the activity of forming a complex in steady state as a result of binding two molecules, such as a substrate and an enzyme or a TF and its target promoter.

Weber's law holds in the linear input dynamic range of a transcriptional circuit, which can be either increased or decreased depending on the binding cooperativity of the reaction. Cooperativity refers to the effect that the first binding interaction has on the probability of the second binding event. Positive cooperativity means that the binding of one molecule to its target site increases the binding affinity for the second molecule. Many commonly used transcriptional repressors, such as LacI, display a positive cooperativity in binding their target DNA [14]. Higher cooperativity leads to a steeper input-output transfer function [176], which in turn narrows the dynamic range of the system and produces an all-or-none response suitable to a digital logic circuit. In contrast, negative cooperativity, whereby the binding of the first molecule lowers the affinity for the second binding, is commonplace in cell signaling networks [177] and it allows a system to respond in analog fashion over a wide input dynamic range.

Building an analog circuit requires a wide dynamic range (the range of input concentrations over which the system displays a linear input–output response). One challenge in constructing analog synthetic

biology circuits lies in the switch-like behavior of the synthetic biology parts that results from the narrow dynamic range of many biological components. In order to build an analog circuit with these components, the circuit topology must be constructed in a way that promotes a wide dynamic range.

Negative feedback loops are commonly used to linearize the input–output transfer functions of electronic and biological systems – that is, to make the input–output transfer function linear over a wider range of input concentrations. For example, one study implemented an autonegative feedback loop to linearize the response of a simple circuit to the small molecule aTc in *S. cerevisiae* [176] (Fig. 9a). The authors compared two circuits. In the first circuit, the TetR repressor is expressed from a constitutive promoter, and it represses transcription of a GFP reporter gene. The addition of aTc relieves transcriptional repression by TetR, allowing GFP expression. The circuit shows a digital response, with a narrow transition region between the GFP-off state (low aTc) and GFP-on state (high aTc). The second circuit is identical to the first, except that TetR is expressed from a TetR-repressed promoter – that is, autonegative feedback is introduced into the circuit. In contrast to the first circuit, the negative feedback loop allows linear GFP expression. A possible explanation is that the autonegative feedback reduces the basal level of the TetR repressor, because at low aTc concentrations the TetR protein represses its own production; this leads to higher basal GFP levels and, consequently, a linear input–output function at low levels of aTc [176]. The autonegative feedback circuit has also been constructed in mammalian cells and has achieved a linear response [11].

Madar *et al.* [178] studied the naturally occurring autonegative feedback loop in the arabinose utilization system of *E. coli* (Fig. 9b). These authors showed that removing the autonegative feedback from the arabinose regulatory network decreased the input dynamic range by 10-fold. The arabinose system is regulated by cAMP receptor protein (CRP), which is activated by cAMP, and AraC TF, which is activated by L-arabinose. AraC also represses its own promoter, creating an autonegative feedback loop (see [179–183] for details). To understand the role of the autonegative feedback loop, the authors put AraC under control of a constitutive promoter (Fig. 9b), and found that a loss of the autonegative feedback decreased the L-arabinose dynamic range by an order of magnitude [178].

Recently, Daniel *et al.* [171] implemented a strong negative feedback loop in a genetic circuit, yielding a power law function relation between the input and the output. The input to this circuit is IPTG, which inhibits the binding of LacI to the P_{LacO} promoter that drives the expression of AraC from a low-copy plasmid. AraC in turn binds to the P_{BAD} promoter located on a high-copy plasmid and activates expression of the LacI repressor when the arabinose concentration is high. A strong and tunable negative feedback loop was achieved by adjusting the ratio between the low-copy and high-copy plasmid (Fig. 9c). The LacI-IPTG transfer function exhibited a power law function ($y = x^{0.7}$) over two orders of magnitude (see Ref. [171] for details), thus enabling power law computations in living cells with few synthetic parts.

A properly tuned positive-feedback loop can also linearize the dose response and extend the input dynamic range of a circuit. Daniel *et al.* [171] implemented

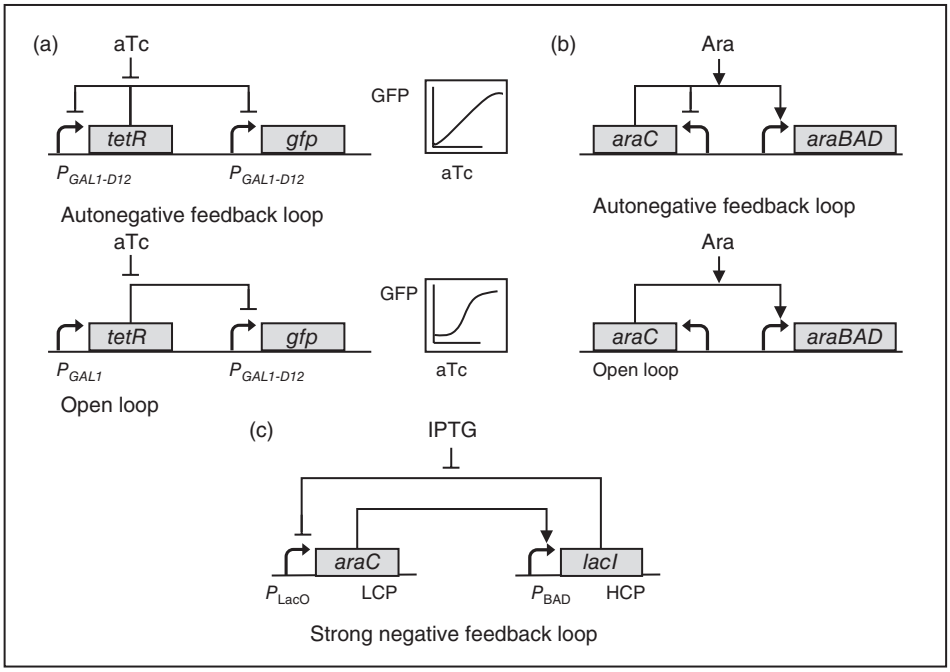

Fig. 9 Negative feedback loops in synthetic biology. (a) Autonegative feedback linearizes the dose response in a simple synthetic circuit [176]. The autonegative feedback loop (top), in which TetR represses its own expression as well as that of the GFP-expressing promoter, shows a graded, linear input–output function, in contrast to the steep input–output function of the open loop (bottom); (b) Autonegative feedback loop in the arabinose utilization system of *E. coli* creates a linear response to increasing concentrations of arabinose. In the open loop system, which lacks negative autoregulation by AraC, the dynamic range is narrower by an order of magnitude compared to the natural system [178]; (c) Strong negative feedback achieves a power law function [171]. HCP, high-copy plasmid; LCP, low-copy plasmid. Panel (a) modified from Ref. [176]; panel (c) modified from Ref. [171].

graded positive feedback loops in *E. coli* for two different types of TF, LuxR and AraC, and their small-molecule inputs (AHL and arabinose, respectively). The input–output transfer functions exhibited a wide region of linearity (over three orders of magnitude) when plotted on a semi-log plot. The circuit consists of two parts: the positive-feedback loop placed on a low-copy plasmid; and decoy binding sites encoded on a high-copy ''shunt'' plasmid, which reduces the positive-feedback loop strength by shunting away a proportion of the TFs that are produced by the positive feedback circuit (Fig. 10a). The shunt prevents the system from saturating at intermediate concentrations of the input, thus extending the dynamic range. When the ''shunt'' circuit was removed, the input–output dynamic range decreased by two to three orders of magnitude [171].

Like digital circuits, analog circuits can also be composed into cascades and more complex devices. The first successful two-stage analog cascade in living cells was used to linearize the P_{lacO} promoter transfer function, which is repressed by LacI [171] (Fig. 10b). The first stage consisted

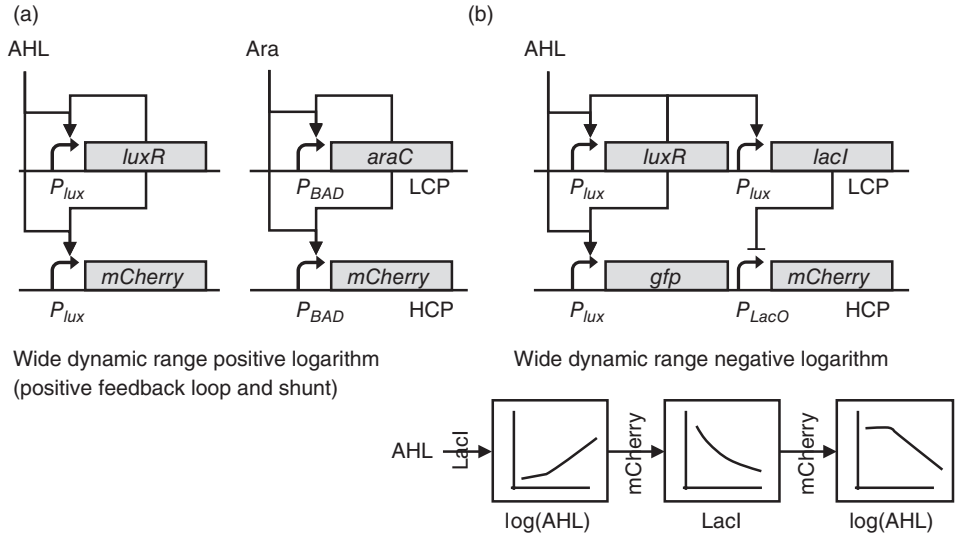

Fig. 10 Positive-feedback loops in analog synthetic biology circuits [171]. (a) Wide-dynamic-range positive-logarithm circuits for AHL and arabinose (Ara). The circuit achieves a wide dynamic range by using a positive-feedback loop and a high-copy plasmid shunt. The positive-feedback loop prevents saturation of the transcription factor at intermediate concentrations of the inducer, and the high-copy plasmid prevents saturation of TF-binding sites. This results in an input-inducer-to-output-protein transfer function that exhibits logarithmically linear behavior with a positive slope; (b) Wide-dynamic-range negative logarithm circuit for AHL. HCP, high-copy plasmid; LCP, low-copy plasmid. Modified from Ref. [171].

of the positive-feedback-and-shunt motif with AHL as the input and LacI as the output, while the second stage consisted of a LacI-repressed P_{LacO} promoter driving the expression of an mCherry fluorescent output from a high-copy number plasmid. The input–output transfer function of the cascade exhibited a wide region of linearity when plotted on a semi-log plot with a negative-slope function that spanned over four orders of magnitude (Fig. 10b).

Finally, combining analog circuits can enable the implementation of complex mathematical functions [171]. An analog adder was built by integrating, in parallel, two wide-dynamic-range positive-logarithm circuits (positive-feedback-and-shunt motifs) that each take in distinct input molecules (AHL and arabinose) and generate an output signal that is common between the two circuits, mCherry (Fig. 11a). An analog ratio-meter was also constructed using the same concept as the analog adder; however, the AHL-responsive positive slope circuit was replaced with an AHL-responsive negative slope (Fig. 11b). The output of the circuit is proportional to the ratio of the inputs (AHL and arabinose). The ratio-meter operates over four orders of magnitude [171]. Circuits capable of calculating the ratio between two inputs are potentially useful, as they enable synthetic biologists to mimic natural biological systems, many of which are balanced between two competing inputs, and to normalize inputs with respect to each other for biosensing and control applications.

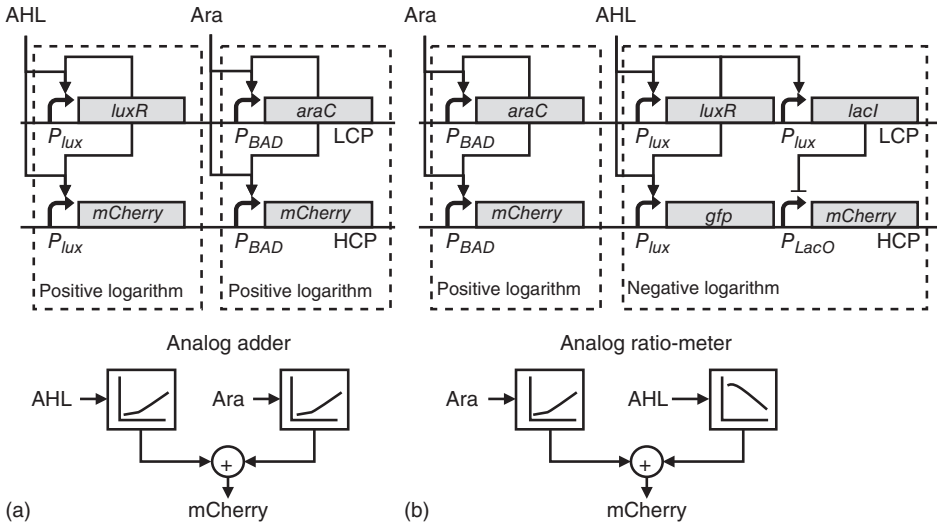

Fig. 11 Analog circuits perform mathematical functions in living cells. (a) An analog adder and (b) an analog ratio-meter were implemented through combinations of positive-logarithm and negative-logarithm circuits that accept distinct inputs (AHL and arabinose) and generate a shared output, mCherry. Modified from Ref. [171].

The design and construction of synthetic analog circuits in living cells is a novel approach that poses new challenges. The accumulation of noise and the need for a high signal-to-noise ratio will be among the main challenges in scaling-up analog genetic circuits. However, these challenges have been faced and addressed by scientists in other fields and their solutions can surely be adapted to synthetic biology. For example, hybrid analog–digital designs can optimize energy efficiency and information precision in one system (see Ref. [171]).

7 Intercellular Communication and Synthetic Multicellular Devices

While the above-described circuits function at the level of a single cell, there is growing interest in programming multicellular systems in which multiple cells communicate with each other to accomplish a specific task [184]. One reason for this is the limitation on the size and complexity of a circuit that can be built in a single cell, due to the crosstalk among circuit components and the metabolic load imposed on the cell (see Sect. 8). Yet, the designers of multicellular systems may overcome this limitation by dividing a large, complex circuit into smaller subsystems that are implemented in separate cell strains programmed to communicate with each other. In addition, populations of cells may carry out behaviors that cannot be implemented in a single cell, such as spatial patterning, thus opening up the possibility of practical applications such as tissue patterning for transplants. Currently, there is also much interest in developing consortia of multiple microbial strains or species that can cooperate in the synthesis of biofuels and other valuable chemicals [185].

7.1 Intercellular Communication Mechanisms

In a multicellular system, cells must be able to send and receive signals among each other. Multicellular synthetic circuits have harnessed the molecules that cells use naturally to communicate. One well-known example is quorum sensing (QS), a process by which bacteria communicate with each other via the diffusion of small molecules that they synthesize. A QS module includes an enzyme that synthesizes the diffusible signaling molecule and a TF that regulates its target genes when bound to the signaling molecule. In *E. coli*, commonly used orthogonal QS systems are LuxR/LuxI from *Vibrio fischeri* and LasR/LasI or RhlR/RhlI from *Pseudomonas aeruginosa* [15, 16]. The LuxI enzyme synthesizes the diffusible signaling molecule, an AHL which, when bound to the LuxR TF, causes gene expression from the P_{lux} promoter. Other forms of intercellular communication exist among eukaryotes: *S. cerevisiae* cells communicate using mating pheromones [186], while mammalian cells possess a suite of signaling proteins that trigger complex signaling cascades and elaborate behaviors in recipient cells [187–192]. Alternatively, a cell may be engineered to synthesize a molecule that does not normally function in signaling, and that molecule may then diffuse out of the sender cell and into a recipient cell engineered to perform a specific action in response to the input.

One disadvantage of small-molecule-based intercellular communication is that only one type of information can be transmitted through a given channel (namely, high versus low concentrations of the signaling molecule) [193]. More advanced multicellular devices could be programmed if the communication channel could transmit diverse user-defined messages. An obvious candidate for such communication channel is intercellularly transmitted DNA. In a recent study [193], communication was engineered between *E. coli* cells via M13 bacteriophages, which exit the host cell without killing it and can transmit multi-kilobase single-stranded DNA molecules between cells. In this proof-of-concept study, the authors programmed sender cells with M13 phages encoding T7 RNAP. Upon receiving the phage message, the receiver cells activate GFP reporter expression from a T7 RNAP-responsive promoter [193]. Another possibility for DNA-based intercellular communication would be to use bacterial conjugation, the process by which bacteria exchange plasmids. While this has not yet been implemented, a recent computational simulation suggests that digital logic gates may be constructed by coculturing bacterial strains that can exchange plasmid-based messages [194].

7.2 Examples of Synthetic Multicellular Systems

Some multicellular systems are isogenic: all cells in the system are programmed with the same genetic circuit, and they communicate with each other to perform a specific task [184]. In an isogenic system, intercellular communication provides a way for cells to synchronize their behavior and to form spatial patterns. In one example, bacterial QS was used to couple and synchronize oscillators (see Sect. 3.1) across a bacterial population [195] (Fig. 12). It should be noted that, at low cellular concentrations, oscillations were not observed as the concentration of the QS molecule

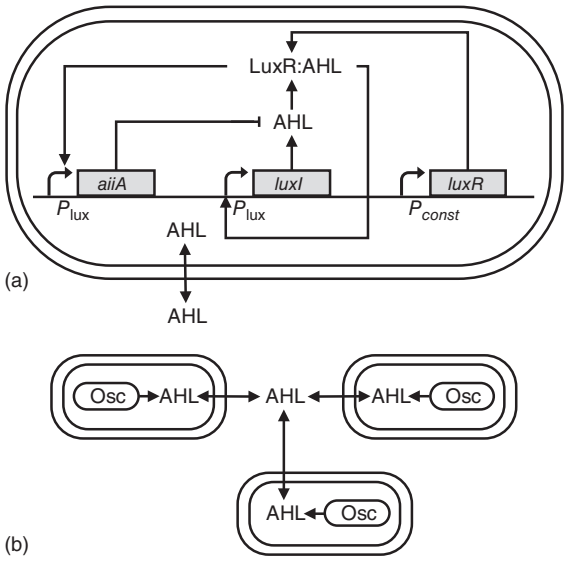

Fig. 12 Using quorum sensing (QS) to couple and synchronize oscillators in an *E. coli* population. (a) Circuit diagram of the synchronized oscillator [195]. AHL is a diffusible QS molecule produced by the LuxI enzyme. The LuxR TF is expressed from a constitutive promoter (P_{const}) and complexes with AHL to make LuxR:AHL, which activates the P_{lux} promoter. AiiA catalyzes the degradation of AHL. As AHL accumulates, more AiiA is produced from the P_{lux} promoter, leading to degradation of AHL; subsequent loss of AiiA expression allows AHL levels to rise again after a delay, leading to oscillations; (b) AHL is used to couple together the dynamics of oscillators in different cells across the population. Osc, oscillators. Panel (a) modified from Ref. [195].

AHL was too low to sufficiently activate gene expression. However, concentrating the bacterial cells in a microfluidic chamber allowed AHL to reach sufficient levels to trigger oscillations [195]. In a related study [196], a collection of contained cellular populations arranged in a grid was synchronized using hydrogen peroxide that had been synthesized by the cells and diffused among the cell populations.

Multicellular systems allow more sophisticated computation than is possible in a single cell, such as spatial patterning in a cell population. An outstanding example is the bacterial edge detection circuit [197]. By coupling a light sensor (Cph8), logic (NOT and AND) gates, and QS modules, the authors programmed a population of *E. coli* cells to detect and outline the (dark-light) edges of a projected image on a lawn of bacteria (Fig. 13). In this example, a cell produces an output (pigment) only if the cell itself is exposed to light whilst its neighbors, which communicate with it via QS, are in the dark [197]. In another study, *E. coli* cells were programmed to form a pattern of alternating stripes of high cell density and low cell density on a culture plate by coupling QS to cell motility [198].

Unlike isogenic cell systems, some multicellular systems consist of two different cell strains, each engineered with a different circuit. Such multistrain consortia can be used for constructing digital logic gates (see Sect. 5). The construction of multilayered logic gates in single cells is hampered by crosstalk among different

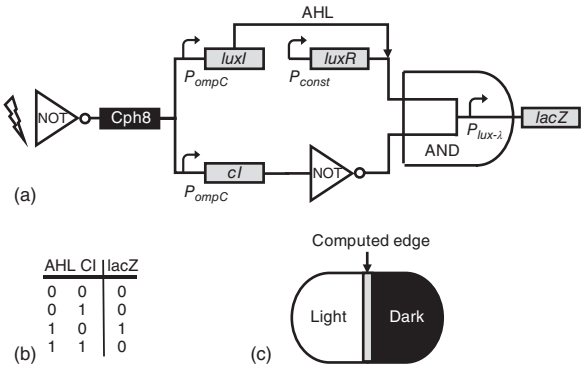

Fig. 13 The bacterial dark/light edge detection circuit in *E. coli* [197]. (a) Cph8 is a light-sensing protein, which can activate the P_{ompc} promoter only in the absence of red light. As a result, only cells in the dark can express LuxI and synthesize AHL molecules, which then diffuse to neighboring cells and allow LuxR to activate expression of the *lacZ* reporter. Cells in the dark also express the λ CI transcription repressor from a P_{ompc} promoter. Only cells that do not express CI and that receive AHL from nearby cells can express *lacZ* from the $P_{luxR}-\lambda$ hybrid promoter. The expression of *lacZ*, which encodes a blue pigment-producing enzyme, occurs only at the edge of light and dark, in cells that receive light (no CI expressed) and are near cells in the dark (receive AHL); (b) Truth table for the circuit shown in panel (a); (c) A schematic representation of the computed dark/light edge (gray) on a lawn of bacteria. Modified from Ref. [197].

parts of the circuit, as well as by the large metabolic load that a large genetic circuit places on the host cell (Sect. 8). Both problems may be circumvented by distributed multicellular computation, whereby each subpopulation processes a specific logic gate and produces output in the form of a diffusible small molecule, which in turn can diffuse through the cell population and act as an input for a logic gate in another cell. Using this distributed computing strategy, Tamsir *et al.* constructed all 16 possible two-input logic gates in *E. coli* [199]. In another example, populations of yeast cells, each encoding an individual logic gate, were connected via diffusible pheromone ''wires'' to make higher-order digital logic circuits, including a multiplexer and 1-bit adder [172]. In both of these complex circuits layering of the gates was made possible through controlled interactions of subpopulations of cells.

The first synthetic intercellular communication device in mammalian cells used sender cells engineered to synthesize the volatile molecule acetaldehyde, which diffused into neighboring receiver cells and triggered reporter gene transcription from an acetaldehyde-responsive promoter [200]. Replacing the mammalian sender cells with acetaldehyde-producing *E. coli* or *S. cerevisiae* cells allowed interspecies signaling [200]. A subsequent study demonstrated two-way communication in mammalian cells, whereby one cell strain produced L-tryptophan and expressed a reporter gene in response to acetaldehyde, while the second cell strain synthesized acetaldehyde and responded to L-tryptophan [201]. Using this bidirectional system, the authors programmed the cell consortium for sequential production of angiopoietin-1 and vascular endothelial growth factor, two proteins required for the formation of mature

blood vessels [201]. This study presents an example of how synthetic intercellular signaling may be used for tissue engineering, with potential therapeutic benefits.

8 Synthetic Circuit Construction: Challenges and Solutions

Synthetic biology aims to make the design of biological devices as predictable as in other disciplines, such as mechanical or electrical engineering. While great progress has been achieved, this goal remains elusive, due to the immense complexity and an incomplete understanding of living organisms. In order for a synthetic circuit to function correctly, the following conditions must be met [202]:

- Each component of the circuit (TF, promoter, regulatory RNA element, and others) should function as expected.
- The circuit design and the parameters of the circuit components (e.g., the rates of TF synthesis and degradation) are suitable to the specified task.
- The circuit avoids unwanted interactions with the host or the host's environment that disrupt circuit function or impair the host's viability.
- The circuit is robust, that is, it is capable of maintaining function in the presence of intrinsic and extrinsic noise.

Synthetic building blocks and their functions are described in Sect. 2. Below are discussed various aspects of circuit design, circuit–host interactions, and robustness to noise. Further discussions of synthetic circuit troubleshooting are available in recent reviews [202, 203].

8.1 Circuit Design: Topology and Parameters

Previous studies in synthetic biology have shown that circuit topology (the way in which the circuit components are wired together) and the parameters of the circuit components qualitatively affect circuit behavior. For example, the damping behavior of an oscillator depends on its topology: a repressilator that consists of two genes repressing each other shows damping after a limited number of cycles, but an oscillator with an amplifier loop shows sustained oscillations [121] (Sect. 3.1). In the case of a toggle switch, changing the relative expression levels of the two transcriptional repressors can change the behavior of the circuit from a switch into a timer [137]. Recently, a gene circuit in *E. coli* was converted from analog to digital function through adjusting plasmid copy numbers with a small-molecule inducer [171].

Computational modeling of different circuit topologies and sets of parameters has been used to identify network motifs that are necessary for, or enriched in, circuits that carry out desired functions. For example, the computational modeling of 1.6×10^8 three-node enzyme-based networks has been carried out to identify networks capable of adaptation (defined as an initial response to a stimulus, followed by return to the initial state even in presence of continued stimulus, a common property of sensory systems) [204]. The authors found that all 395 networks that display robust adaptation shared either one or two of the following motifs: a negative feedback loop or an incoherent feedforward loop (see Sect. 8.3) [204]. In another study, Lim and colleagues used computational modeling to identify candidate network motifs that can induce cellular polarization, a vital property of living

cells that is a prerequisite to behaviors such as directional migration [205]. These authors used the predictions to construct synthetic networks that trigger asymmetric accumulation of the membrane phospholipid PIP3 in the cell membrane of *S. cerevisiae* cells. Robust polarization was accomplished using networks that combined positive feedback with mutual inhibition between the synthetic proteins [205].

Another important consideration in circuit design includes level matching: in any circuit where the output of one part of the circuit serves as the input for another part, the concentration of the output must be in the correct range to trigger the desired response from the downstream part of the circuit. Level matching may be achieved in different ways, such as adjusting the copy number of a gene, or mutating its RBS to affect translation initiation rates and hence the level of protein expression [30].

Moreover, the time required for each layer of the circuit to process its input and produce an output must be considered [206]. The time delay due to biological processes such as transcription and translation affects the timing of its response, and may limit the complexity of circuits that can be implemented in living cells. For example, RNA-based cascades, which do not require translation, are faster than TF-based regulatory cascades [87]. Protein-based signal transduction pathways are faster still, but the ability to design them is still rudimentary despite advances made over the past few years [118].

The response time of a transcription regulator may be shortened by using a negative autoregulatory feedback loop, in which the TF represses its own promoter. This strategy relies on using a strong promoter, which drives TF expression at a high level when initially induced. When the TF has accumulated above a threshold it binds and represses its own promoter. This combination of a rapid initial build-up in TF concentration followed by negative autoregulation allows the TF to reach its steady-state level very quickly [207].

In order to design a circuit with the desired behavior, synthetic biologists take advantage of mathematical modeling. Commonly used mathematical modeling techniques include sensitivity analysis, which quantifies the effect of a parameter (or parameters) on overall circuit performance, and bifurcation analysis, which identifies the boundary in parameter space that separates circuits with qualitatively different behaviors, such as a stable steady state versus an oscillator [208]. Multiple software programs are available for modeling circuit behavior *in silico* (for reviews, see Refs [162] and [208]). As circuits become more complex, however, reliance on mathematical models for their design will increase. For example, Purcell *et al.* recently described a platform for simulating synthetic circuits in the context of whole-cell models [209].

8.2 Circuit–Host and Circuit–Environment Interactions

Unwanted interactions between the circuit and its host cell may lead to circuit failure or cell death. For example, a component of the circuit may be toxic to the host cell and indeed, a recent study identified over 15 000 heterologous genes as toxic to *E. coli* [210]. Conversely, endogenous host genes or proteins may interfere with circuit function; for example, a native DNA sequence may bind a sTF and titrate it away from its target promoter. An increased knowledge of the host cell's regulatory and

metabolic networks through the analysis of large-scale datasets will help synthetic biologists in designing circuits that will function as expected in a given host.

Even when none of the circuit components is toxic to the host, the designers must consider the metabolic load that the circuit imposes on the host cell in terms of requirement for ATP, RNAPs, ribosomes, and nucleotides [211, 212]. A metabolically expensive circuit may interfere with the cell's normal function and place selective pressure on the host to inactivate the synthetic device.

Certain circuit designs may help to alleviate metabolic load. Different ways of implementing the same type of function may have different energy requirements; for example, a memory device based on one-time recombinase-mediated DNA inversion is less metabolically demanding than a memory device that requires continued protein synthesis [153]. Moreover, regulatory RNA devices, which do not rely on the host's translation machinery, have a smaller metabolic footprint than TF-mediated regulation [87]. Another way to reduce the metabolic burden on any single cell is to distribute a large circuit among several strains of cells by placing a smaller subcircuit in each cell strain [172, 199] (Sect. 7). While powerful, distributed computing adds a level of complexity to the circuit design; the investigator must consider not only the effect of the circuit on each host strain but also the way in which two or more host strains interact to accomplish their task. For example, if cells of different strains communicate via a diffusible molecule, they must be in close physical proximity in order for the receiver cell to detect the signal [199]. If distributed computing is carried out by multiple engineered strains in a common culture medium [172], the computation may fail if one of the strains outcompetes the others.

8.3 Noise and Robustness

Living cells are noisy, due to differences among individual cells (or in a single cell over time) in parameters such as the number of TFs, mRNAs, and ribosomes; cell volume; state of cell cycle; and chromatin modifications [213]. Every component of a genetic circuit – whether synthetic or natural – experiences and propagates this noise to some extent, which in turn can be amplified by the noise from other components of the circuit. This will intensify the overall noise and hence may disrupt the performance of a genetic circuit. Moreover, the cell must cope with extrinsic noise, such as fluctuations in nutrient availability, temperature, pH, and other environmental variables. Notably, many practical applications (e.g., medicine or bioremediation) will require the engineered cell to function in a more unpredictable environment than a culture flask in the laboratory. Hence, a synthetic device must be able to minimize noise where possible and to maintain a desired function in the presence of unavoidable noise; this property is called *robustness* [214].

Over long time scales, evolution has selected for robustness in natural gene circuits [215]. However, in synthetic circuits, which lack an evolutionary tuning process, noise can blur the desired output and therefore is not usually considered a favorable factor. Without a mechanism for controlling noise, increasing the complexity of a circuit will likely increase the uncertainty of the output.

The noisy nature of biological systems is part of the reason why many synthetic

circuits employ digital logic (see Sect. 5). Digital designs reduce the effect of noise by reducing the number of outputs of a given component of a circuit to two states: TRUE and FALSE. Ideally, the states are defined in such a way that passing from one state to another requires a significant change in concentration of input(s) of the component (e.g., an inducer or a TF), and therefore transition between states by cellular noise alone is very unlikely. Given that the number of distinguishable states that a circuit can possess can be considered a measure of complexity of the circuit, reduced complexity is a price that digital designs pay to overcome cellular noise. Digital gene circuits are generally more robust with respect to cellular noise than their corresponding analog circuits; however, they require more components than an analog circuit to achieve the same level of complexity, and hence they place a higher metabolic load on the cell [216].

Computational and experimental analysis of naturally occurring gene network motifs has revealed the robustness of certain motifs. For example, negative autoregulation, whereby a TF represses transcription of its own gene, helps to minimize noise in the TF expression levels: cells that initially have more TF will produce less of it, and cells that initially have less TF will produce more [217]. Another example of a motif that occurs commonly in natural gene regulatory networks is the feedforward loop (FFL), which consists of three nodes: node X regulates node Y, and X and Y regulate node Z. In coherent FFLs, the regulatory interaction (either activating or repressing) between X and Z is the same for both branches of the regulatory pathway; that is, if X activates Z directly, then X also activates Z via Y. In an incoherent FFL, the direction of the regulatory interaction is different for the two branches of the pathway so that, for example, X activates Z directly and inhibits Z via Y [218]. Mathematical modeling indicates that the coherent type 1 FFL, which consists entirely of positive interactions – X activates Y, and X and Y both activate Z – is the most robust to noise among the coherent FFLs [219]. The robustness of the coherent type 1 FFL may account for the large number of times this motif occurs in the gene regulatory networks of *E. coli* and *S. cerevisiae* [219]. Synthetic circuit design may benefit from using robust genetic motifs such as the coherent type 1 FFL that occur repeatedly in many natural systems, providing them with properties such as fast response time, robustness, or memory (see Ref. [218] for a detailed discussion of network motifs).

8.4 Evolution

In addition to making a system robust to short-term fluctuations in the levels of metabolites, circuit components, and so forth, synthetic biologists must also consider the impact of evolution on the function of the circuit over time. While evolution has been harnessed to improve circuit function and develop libraries of diverse components [220], it is also problematic in synthetic biology: circuits undergo point mutations, rearrangements, and deletions that disrupt their function, and may be lost from the host cell altogether after a number of generations. Repetitive sequences, high metabolic load, high plasmid copy number, and a quickly replicating host cell such as *E. coli* increase the probability of the circuit being lost or mutated [214].

The issues of circuit–host interactions, robustness and evolution are interrelated (e.g., a high metabolic load is likely to lead

to evolutionary instability of the circuit), and may present trade-offs. For example, a reduction in protein level noise appears to come at the cost of higher energy requirements [221]. Synthetic biology is an iterative process: successes and failures in circuit construction teach about the mechanisms of gene network function, and these lessons can then be applied to the next round of circuit modeling and design. A deeper understanding of living systems, larger and better-characterized component libraries, and better models will help synthetic biology to advance the construction of predictable, robust circuits for practical applications.

9
Applications of Synthetic Circuits

Synthetic gene circuits used in applications must function reliably in adverse and often undetermined conditions. Consequently, applications push the field forward by challenging synthetic biologists to design robust, efficient circuits [7]. Examples of three types of practical applications – biosensors, therapeutics, and biomanufacturing – are presented below.

9.1
Biosensors

Biosensors translate the concentration of a specific analyte in the environment into a measurable signal by combining a living cell with a hardware platform that enables detection [222]. The output is usually a measurable signal, such as GFP or the blue pigment produced by the β-galactosidase enzyme. Biosensors may be judged by their selectivity for binding the target analyte, their sensitivity for low concentrations of the analyte, the input dynamic range of analyte concentrations, and the output signal-to-noise ratio. Many biosensors use transcription-factor-based circuits to measure the concentration of a toxic compound such as arsenite [196, 223, 224]. The goal is to use these biosensors in the developing world for testing water before its consumption. These field conditions make standardized measurements challenging, because the physiological state of the living cells in the biosensor is highly variable. To overcome this, Wackwitz *et al.* [224] developed a series of complementary cell strains that are tuned to respond to different arsenite concentrations by changing the strength of the RBS in front of the reporter gene. When calibrated and used in concert, these strains greatly improved arsenite detection. Rather than having the steady-state expression level of a reporter protein as their output, Hasty and colleagues [196] developed an oscillator whose frequency varies as a function of arsenite concentration; this decoupled the biosensor from the imaging conditions, such as beam power and exposure time. Further, the biosensor was an array of bacterial colonies in a microfluidic device that synchronized their oscillations at both the micro and macro-scale through diffusible and gaseous vapor molecules, respectively, enabling an accurate, high-strength signal that allows the biosensor to function as a handheld device.

In addition to cellular biosensors, synthetic biology has enabled the development of real-world deployable microbial sensors based on engineered phages. For example, Sample6 Technologies is commercializing a near-real-time microbial pathogen detection system based on engineered phages [225]. Bacteriophages (''bacteria eaters'') are viruses that infect specific species and strains of bacteria [226]. By engineering phage libraries to express

reporter genes during the infection of target bacteria, the presence or absence of pathogens can be detected. Reporter phages have been designed for the detection of clinically relevant pathogens such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and food-borne pathogenic *E. coli* [227]. The advantages of engineered phage diagnostics include high sensitivity, high specificity, and short time-to-detection, which are shortcomings that are not addressable by conventional microbial detection approaches such as polymerase chain reaction (PCR), immunoassays, and culture. Moreover, phages specifically detect live bacteria that support phage replication, whereas some other rapid detection methods, such as PCR, do not distinguish between live and dead bacteria [227]. The tools of synthetic biology enable a rapid design–build–test cycle for new diagnostic systems, such as engineered phages, thus allowing assays to be built, tested and improved for real-world applications.

9.2 Therapeutic Applications

Synthetic biology holds tremendous promise in the area of drug development, diagnosis and treatment of disease [13]. Cell-based therapeutics constitute an emerging therapeutic class, and central to their efficacy are synthetic gene circuits that process environmental signals and actuate treatment, enabling appropriate selectivity, distribution, and dosage [13]. Microbial cells have been engineered to combat infectious diseases by either killing [228] or downregulating the growth [229] of pathogenic organisms. Both therapeutics rely on QS circuits (see Sect. 7) that trigger the release of active agents only in the presence of the target pathogen's quorum signal. Such targeted killing is a promising application for synthetic biology, as antibiotic resistance becomes more prevalent and broad-spectrum antibiotics lose favor. Microbes have also been engineered to invade cancer cells [166] using a synthetic gene circuit that triggers invasion only when the cells reach a critical density (detected via QS), or when they sense the hypoxic environment that is a hallmark of cancer cells with hyperactive metabolism. Furthermore, microbial-based therapies are an increasingly promising area of research as more information is gleaned about the microbiome and the role that symbiotic microorganisms play in human physiology.

Other studies have focused on developing mammalian cell-based therapeutics. Engineered cells may be enclosed in a semipermeable capsule, which allows them to be implanted in the body and to interface with human physiology, while at the same time being isolated from direct contact with the patient's tissues to prevent an immune response or metastasis. This microencapsulation technique was used in a system for regulating urate homeostasis to combat tumor lysis syndrome and gout, two diseases caused by abnormally high urate levels [24]. A synthetic gene circuit in the mammalian cells within the microcapsule utilizes a bacterial TF to sense urate concentrations and control the expression of an enzyme that degrades urate. This system restored urate homeostasis in a mouse model of acute hyperuricemia [24]. A similar study utilized a light-inducible gene circuit to control the production of glucagon-like peptide 1 and reduce glycemic excursions in type II diabetic mice [160].

A T-cell-based system represents another form of emerging mammalian

cell-based therapeutic. In this case, the immune cells are removed from a patient, genetically engineered to target pathogens or cancer cells, and then transferred back into the patient. However, these therapies suffer from side effects such as hyperactivity and autoimmune off-target attacks, and would benefit from a synthetic control of proliferation. Two pioneering studies have demonstrated control over T-cell proliferation with synthetic RNA-mediated [230] or signaling protein-mediated [119] regulation of T-cell replication.

Synthetic gene circuits can also target therapeutics to different cell types via classifier circuits. Two examples demonstrate this approach to target cancer cells. In the first example, Nissim and Bar-Ziv [22] constructed a transcription-based AND gate that only expresses its therapeutic payload in cells in which both input promoters are on. In this design, two input promoters active in a specific cancer cell type are used to drive the expression of two fusion proteins, which form a transcription activator complex that activates the expression of a cytotoxic effector protein. Different input promoters may be chosen to target different cancer cell types. The use of two input promoters rather than one allows greater flexibility in the choice of input promoters, and produces a sharp activation threshold between premalignant and cancer cells, with the magnitude of the response increasing in more malignant cell lines. In order to avoid unwanted activation of the circuit in healthy cells, the level of effector gene expression may be tuned using point mutations in one of the fusion proteins to lower the efficiency of the synthetic TF complex formation. The digital logic circuit provides a precise and efficient way to target specific cell types while minimizing off-target effects on healthy cells [22]. In a second example, Xie *et al*. [23] built a classifier circuit that determines whether the levels of six different miRNAs match the reference profile of cancer cell miRNA expression, and based on this information controls expression of the BAX protein, which triggers apoptosis (see Sect. 5 and Fig. 7). Such deliverable cell-based classifiers utilizing synthetic gene circuits will play an important role in targeting therapeutics for difficult problems such as cancer or gene therapy.

In other examples of cancer therapy, oncolytic viruses were optimized for cancer targeting with improved specificity [156, 231]. Recently, synthetic constructs linking diphtheria-toxin gene expression under the control of the H19 promoter were tested in human patients [232].

Synthetic biology may also be applied to combating antibiotic-resistant bacteria, which present an emergent health threat worldwide [233]. The shortage of effective new antibiotics [234] necessitates the development of novel therapies. One possibility is to use synthetic biology to engineer phages (viruses that naturally infect bacteria) to combat antibiotic-resistant bacterial strains [235]. The engineered phages may be used to target biofilms, surface-associated bacterial communities encased in an extracellular matrix of polysaccharides and proteins that protects the bacteria from antibiotics and from the patient's immune system [236]. For example, T7 bacteriophage engineered to express Dispersin B, an enzyme that hydrolyzes a key biofilm component, efficiently disrupts *E. coli* biofilms [237].

Phages may also supplement antibiotic therapy. In one study, M13 phages were engineered to overexpress genes predicted to make host cells more vulnerable to antibiotics; these genes included *lexA3*, which inhibits the bacterial DNA damage

response, leaving the cell vulnerable to DNA damage-inducing antibiotics; *csrA*, which inhibits biofilm formation; and *ompF*, which encodes a membrane protein through which antibiotics can enter the cell [238]. A combination of each engineered phage and antibiotic kills *E. coli* significantly more efficiently than either a combination of antibiotic and control (unmodified) phage or antibiotic alone [238]. Notably, M13 phage does not kill the host cell in absence of antibiotics, and hence it is less likely to give rise to bacterial resistance, which presents an important problem in antibacterial therapy [235]. In addition to resistance, many other challenges remain on the road to effective phage-based therapy, including potential side effects, the need for the phage to evade the patient's immune system, and a limited phage host range [235]. Nonetheless, the studies described above suggest that phage-based therapy is a promising strategy to pursue in combating antibiotic-resistant bacteria [235].

Finally, synthetic circuits can be utilized for the discovery and optimization of novel pharmaceuticals, such as new antituberculosis compounds [239] and novel classes of treatments for antibiotic-resistant-bacteria, such as lysins (phage-derived proteins that lyse bacteria) or bacteriocins (small peptides that kill bacteria by forming pores in their cell membranes) [233]. Synthetic biology also holds the potential for improving the yield of biopharmaceuticals and other valuable chemicals, as described below [233].

9.3 Synthetic Biology in Manufacturing

Synthetic biology has revolutionized industrial biotechnology by allowing engineers to optimize living cells rationally for the production of pharmaceuticals and other valuable chemicals [240]. Central to these efforts is repurposing biosynthetic genes from various organisms and tuning their level of gene expression. Often, gene expression is engineered to be static, so that it is constant throughout the course of production, or it is controlled with a simple gene circuit, in which an externally added small molecule induces a constitutively expressed TF to switch on the biosynthetic genes. However, these systems suffer from the metabolic burden that they exert on cells, slowing cellular growth and thus impairing productivity. Complex biosynthetic systems would benefit from the dynamic regulation enabled by synthetic gene circuits, wherein the expression of biosynthetic genes is adjusted based on a cell's physiological state, allowing adjustments based on the concentration of pathway intermediates or environmental conditions in the bioreactor such as nutrient availability, oxygen level, temperature, and cell-density [241]. Such dynamic regulation is akin to how cells naturally adjust their own physiology, and is expected to improve culture growth rates.

In an early example of dynamic regulation, the yield of lycopene in *E. coli* was improved by placing the expression of rate-limiting lycopene biosynthetic genes under the control of a TF that sensed excess glucose concentrations [242]. As a result, the pathway was only turned on when the cell had enough energy to continue growing, and this led to an 18-fold higher lycopene production. More recently, Zhang *et al.* engineered circuits to control biosynthetic pathways using an approach called "*dynamic sensor-regulator system*" (DSRS) [243]. These circuits are based on TFs that bind pathway intermediates, and affect synthetic promoters so

that the expression of biosynthetic genes is activated only when needed, at the level needed. Such an approach to biosynthetic gene regulation is theoretically applicable to many different pathways, given the number of known metabolite-binding TFs, and could be further expanded with the use of synthetic aptamers evolved to bind different molecules of interest (see Sect. 2.5). An alternative method of improving the yield would be to tie the regulation of biosynthetic genes to the density of cells in the bioreactor in order to maximize cell growth before activating biosynthesis. This approach has been implemented with a QS circuit as an input to a toggle switch, and it has improved the yield of both recombinant proteins [139] and metabolic molecules [244, 245].

10 Conclusions

The past two decades of synthetic biology have led to a tremendous explosion in the design of ever more powerful and complex synthetic gene circuits. These circuits confer a greater degree of control over engineered biological systems than was ever possible before. However, significant challenges remain in the design and application of synthetic gene circuits due to incomplete biological knowledge, slow design–build–test cycles, nonpredictive *in-silico* models, challenges in designing circuits that can function reliably in different contexts from the laboratory environment in which they were engineered [246], and outstanding issues concerning orthogonality, modularity and noise (for reviews, see Refs [202] and [203]). Yet, synthetic gene circuits clearly have much to offer society, and advances in fundamental circuit design and their implementation in biosensing, medicine, biomanufacturing and other application areas are expected to continue substantially during the next decade.

Note Added in Proof

While this chapter was under review, additional articles on synthetic gene circuits have been published. Chen *et al.* [247] programmed *E. coli* for inducible production of amyloid protein-based extracellular fibrils carrying affinity tags for inorganic nanoparticles. As proof of principle, the authors demonstrated inducible formation of a conductive biofilm composed of proteins decorated with gold nanoparticles. The system shows potential future use of synthetic biology for the formation of materials that combine the properties of living systems and inorganic matter to achieve novel functions [247]. Another recent study combined CRISPR gRNA and RNAi for tunable, multiplexed regulation of transcription in mammalian cells [248]. Notably, the study was the first to achieve inducible synthesis of gRNAs, opening up novel possibilities for inducible regulation of target genes by CRISPR [248]. In addition, a detailed protocol has been published for combining recombinase-based digital logic and memory in living cells [249], and two new reviews discuss features and potential applications of digital vs. analog synthetic gene circuits [250, 251].

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